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MALES OF THE WOLF SPIDER RABIDOSA RABIDA USE TWO MECHANISMS TO STUN FEMALES DURING COPULATION

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MALES OF THE WOLF SPIDER *RABIDOSA RABIDA* USE TWO MECHANISMS TO STUN FEMALES DURING COPULATION

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ABSTRACT

The differences in energetic input between the sexes required to produce gametes translates to the differences in reproductive behavior and overall mating systems seen in a species. Females generally produce a few energetically and resource expensive eggs and typically choose a high-quality suitor to ensure she has high quality offspring. In contrast, males produce abundant energetically cheap sperm and attempt to fertilize as many eggs as possible in as many females as possible. Both sexes are trying to maximize their inclusive fitness, but the dichotomy of interests can lead to sexual conflict and perhaps extreme or unusual behaviors such as sexual cannibalism or manipulation of a mate. However, occasionally, the sexes evolve to help increase each other's fitness during mating, known as sexual cooperation. In spiders, sexual cannibalism of the male by the female is a common occurrence and males of some species have evolved behavioral, morphological, and physiological adaptations to avoid being cannibalized during courtship and copulation. Female *Rabidosa rabida*, a wolf spider, attack their male partners often during the courtship and copulation process but can be left in a quiescent, or stunned, state post-copulation where they remain unresponsive to external stimuli after the male moves away. Behavioral and microscopy studies with other spiders suggest the quiescent state could be induced by a male produced pheromone from cuticular structures on his legs (transferred by either direct contact or volatile transmission), a chemical in the male ejaculate transferred during insemination, or a component of the male's venom that he injects in the female. Using

R. rabida, I investigated proximate and ultimate questions about male induced female quiescence to avoid sexual cannibalism where I used scanning electron microscopy (SEM), mating trials with modified/ablated males, male homogenate trials, and gas chromatography-mass spectrometry (GC-MS). Specifically, I aimed to locate the organ of compound production (SEM and mating trials), to determine whether the compound was transferred directly or if it was airborne (male homogenate trials), and to identify the compound (GC-MS). I found *R. rabida* wolf spiders have cuticular structures on their legs that are presumed to be associated with semiochemical emitting organs. Males likely use these organs to induce a quiescent state in their female mates, and females attack the males less often when quiescent. I also found a variety of lipids, hormones, fatty acids, and other hydrocarbon molecules from the two sexes at different life stages with GC-MS. The compound in question was not identified.

Table of Contents

LIST OF FIGURES

FIGURE

LIST OF TABLES

INTRODUCTION

Adults of some sexually reproducing animals are under selection to maximize evolutionary fitness. Direct fitness is quantified by the number of offspring produced, while indirect fitness includes the number of offspring produced by the individual's offspring or related individuals. The sexes can differ in how each can maximize their inclusive (direct + indirect) fitness which begins at the cellular level and the energetic input to produce gametes – eggs and sperm (Andersson 1994). Anisogamy is the dichotomy between egg and sperm size that leads to females producing a limited quantity of eggs and males producing large quantities of sperm (Andersson 1994). Female eggs are more energetically expensive to produce because they are larger and contain DNA, lipids, proteins, and carbohydrates needed for developing offspring (Andersson 1994). Male sperm are less energetically expensive to produce because they only contain DNA from the sire and just enough energy stores to travel to the egg, thus allowing them to be very small (Andersson 1994). This key sexual difference, carries over into reproductive behavior, including courtship and mating where the motivations of the sexes diverge. In most animal species, females are selective about the males that fertilize their expensive eggs, while males compete to fertilize as many eggs (in as many females) as they can with their abundant sperm (Andersson 1994). In most cases when animals reproduce, both individuals increase their lifetime reproductive success and evolutionary fitness even if their optimal choice of the quality or quantity of mates was not achieved (Andersson 1994). In the case that both individuals' reproductive success

increases, the sexes are cooperating to increase fitness. In some species, males have even evolved mating strategies to benefit their female partners either directly or indirectly. These strategies usually involve the male transferring chemicals or nutrients to the female that result in an increased number of offspring, increased vitality of the offspring, or increased vitality of the female (butterflies: Andersson et al. 2000, Andersson et al. 2003, field crickets: Wagner et al. 2001, Wagner & Harper 2003). Using female *G. lineaticeps*, a field cricket, in a series of single and recurring mating trials with either one male (single mating control and repeated mating treatment) or multiple males (multiple mating treatment) Wagner et al. (2001) found the females gain benefits from male seminal fluids and the benefits increase with additional matings. Specifically, females that mated more than once lived 32% longer than females that mated once and females mated with multiple males gained the additional benefit of producing 98% more eggs than single mating females.

Although the optimal number and quality of mates may differ between the sexes, males and females usually have a net gain of fitness with each copulation (Trivers 1972). Typically, males increase reproductive success and fitness by mating with multiple females while females benefit from selecting high-quality males, known as Bateman's principle (Bateman 1948, Trivers 1972, Wade & Shuster 2005). In contrast, there are mating systems in which the fitness of one sex is decreased at the expense of the other (Parker 1979, 2006). Sexual conflict is far less common among animals, but a few examples have come from insects such as the toxic sperm of fruit flies, in which male seminal chemicals reduce the female lifespan (Chapman et al. 1995), in the bedbug

wherein males injure females by stabbing her abdomen to gain access to her unfertilized eggs (Stutt & Siva-Jothy 2001), and in butterflies where males reduce female chances of remating with pheromones (Andersson et al. 2004). Sexual conflict can also cause the loss of male fitness if the female is aggressive and attacks the male. In some systems, if an aggressive female injures the male, he may lose mating opportunities due to being less mobile (Amaya et al. 2001) or having become a lower quality male (Uetz et al. 1996). Further, in the circumstance that the male being consumed does not increase the brood size or vitality, he cannot gain fitness benefits if being cannibalized is a possibility.

Sexual cannibalism – the consumption of a mating partner prior to, during, or after copulation – is extremely rare in the animal kingdom but relatively common among spiders (Elgar 1992). Whether the cannibalism of a mate is sexual conflict or cooperation depends on the mating system. In some spider species the mating system has evolved to include sexual cannibalism of the male as cooperation between the sexes. This cooperation has evolved to the point where the male will facilitate his own consumption. The males of two species of widow spider (Family Theridiidae), for instance, will offer themselves as a nuptial gift to their female mate during copulation (redback spider, *Latrodectus hasseltii*: Andrade 1996; brown widow, *L. geometricus*: Segoli et al. 2008). This self-sacrifice behavior increases the duration of copulation allowing the male to fertilize more eggs, therefore increasing the number of offspring, and fitness, for both sexes. Further, males of the fishing spider *Dolomedes tenebrosus* have evolved a mating system in which males die 100% of the time with the first ejaculate transfer. Shortly after he dies, the female will grasp the male and consume

him, gaining fitness benefits for both partners such as increased size and vitality of offspring (Schwartz et al. 2013, 2014, 2016).

In many other species sexual cannibalism reflects sexual conflict. In spiders, females are typically the aggressors between the sexes and have a greater influence on conflict outcome. After a roving mature male locates a female, he will court the female with a series of species-specific leg waves and vibrations while the female assesses her suitor's quality (Hebets & Papaj 2005). Throughout this process, the female may reject the male by attacking or cannibalizing him (Elgar 1992, Elgar & Schneider 2004). The possibility of cannibalism remains even if the male successfully courts and mates with the female. Either scenario, if concluding in cannibalism, can increase the fitness of the female spider, i.e., she gains nutrition and reproduces, while decreasing the males' potential direct fitness since he cannot mate with more females. This sexual conflict by sexual cannibalism is considered one of the most extreme variations of sexual conflict (Schneider 2014).

In some spider species the males have developed cannibalism avoidance strategies in response to the pressures from aggressive and cannibalistic females. One of the most common methods for male spiders to avoid becoming prey, is to perform a stereotyped, species-specific courtship display (Hebets & Papaj 2005). By signaling its species identification and potentially, mate quality, the female may be motivated to mate, rather than to eat (Hebets & Papaj 2005). These displays also function in mate choice by females and include multiple signals and sensory modalities (Hebets & Papaj 2005).

Additional tactics used to decrease female aggression include distraction, physical restraint, and chemical transfer of pheromones, venoms, and ejaculate. Nuptial gifts, typically a prey item captured by the male, may be presented to the female to shield the male from precopulatory cannibalism (*Pisaura mirabilis*: Toft & Albo 2016) by exploiting the female's senses and foraging motivation (Stålhandske 2002, Albo et al. 2017, but Bilde et al. 2007 suggest it is only foraging motivation). In one species, *P. mirabilis*, this distracting, exploitative behavior is occasionally accompanied by the male entering thanatosis, or feigning death, when courting aggressive females (Bilde, et. al. 2006). Shortly after the female begins eating the gift, the male will 'revive' himself and begin copulation.

Males of other species have evolved more straightforward behaviors and morphological characteristics that physically restrain aggressive females instead of distracting her. Many mygalomorph spiders (e.g. tarantulas, purseweb spiders, tunnel web spiders) mate in an upright position, ventral sides together, with the male propping up the female so her sternum is exposed (Jackson & Pollard 1990). In this position her fangs are directly above the male, an easy position to attack and kill the male. The male uses a clasper, an enlarged tibia and curved metatarsus, to restrain the female in mating position presumably to avoid being attacked and cannibalized (Jackson & Pollard 1990). However, this behavior may also be used to recognize and communicate with mates (Jackson & Pollard 1990, reviewed in Ferretti et al. 2013). Interestingly, female *Porrhothele antipodiana*, and other mygalomorph spiders, enter a quiescent state after the males clasp them during courtship (Jackson & Pollard 1990, Ferretti et al. 2013).

Female *P. antipodiana* became passive for up to five minutes after removing the male mid-copulation and did not resume an active state even when pushed or lifted (Jackson & Pollard 1990).

Males of many other spider species have been found to deposit silk on their mates with a variety of possible uses including cannibalism avoidance (reviewed in Scott et al. 2018). Binding cannibalistic females with silk has been shown to allow male *Pisaurina mira,* nursery web spiders, to escape cannibalism. Anderson and Hebets (2016) ablated the spinerettes of male *P. mira* and found males were significantly more likely to be cannibalized when unable to bind the female with silk. This behavior is likely the response to sexual conflict as they also found females gained fitness benefits from cannibalizing their mates. The offspring had higher survivability if the male was consumed in comparison to the female consuming a cricket or nothing (Anderson & Hebets 2018).

Other hypotheses about silk wrapping behavior include it being used as a substrate to transfer semiochemicals to the female. The use of silk laden with semiochemicals (e.g. pheromones) by male spiders has been hypothesized many times but lacks compelling evidence, unlike the semiochemical laden silk of their conspecifics (reviewed in Fischer 2019). However, there is behavioral evidence for male produced pheromones for two sex-role reversed wolf spiders (Aisenberg et al. 2010). Males are known to locate females via pheromones. In some species, males deconstruct female's webs to avoid competition by other males, and to assess the female's quality and mating status (*Pardosa milvina*: Rypstra et al. 2003). Hypotheses involving male

produced semiochemicals include them being used as aphrodisiacs, anti-aphrodisiacs, aggression reducers, and catalepsy/quiescence-inducers (reviewed in Fischer 2019).

Semiochemical use by males for female manipulation has been observed in several species of spiders. Only one male semiochemical, an aphrodisiac, has been identified to date (Xiao et al. 2010, Fischer 2019). Xiao and colleagues (2010) used a combination of gas chromatography-mass spectrometry (GC-MS), behavioral assays, and electroantennography to determine the compound and behavioral effects of the compound with male *Pholcus beigingensis*, a cellar spider. They found the males use (Z)-9-tricoscene, an alkene, to initiate copulation sooner and it was not used as an attractant. Males of other spider species produce secretions from protuberances and grooves on their cephalothoraxes that are hypothesized to function as aphrodisiacs (*Argyrodes spp.*: Whitehouse 1987, *Diplocephalus permixtus*: Uhl & Maelfait 2008). The secretions may also function as a male produced nuptial gift (*Hedypsilus culicinus*: Huber 1997, *Oedothorax spp.*: Kunz et al. 2012, Kunz et al. 2013) and do not influence the female's receptivity but the functions of and specific compound produced for each of these species has not yet been examined.

Anti-aphrodisiacs are used to manipulate a female to reduce her receptivity to additional males or to reduce her attractiveness to other males after mating. The former use of an anti-aphrodisiac pheromone has been suggested for the wolf spider *Schizocosa malitiosa* (Aisenberg & Costa 2005). Asienberg and Costa (2005) prevented newly matured male *S. malitiosa* from filling their haematodochal sacs with sperm, known as charging their pedipalps, following maturation. They then introduced these

and control males to females in a series of mating trials. The females that mated with control males were not receptive to additional matings three days after their initial mating. Females that mated with males that did not charge their pedipalps were receptive to additional matings three days after the first mating despite normal courtship and copulation behaviors and any other possible substance transfer. On the other hand, males may mask the attractiveness of a female by masking her pheromones with their own thus deterring future suitors (Scott et al. 2015).

Web reduction behavior, where males deconstruct receptive females' webs has been shown to reduce female pheromone dispersion and lower the chances of additional males finding her (*Neriene litigiosa*: Watson 1986). Males of some spider species will add their own silk to the deconstructed female web. While the added silk may act simply as a physical barrier, it may also include a male produced anti-aphrodisiac used to deter future male suitors by reducing the attractiveness of the female. This male deterring tactic is hypothesized to be utilized by males of the widow spider *Latrodectus hesperus* (Scott et al. 2015). This tactic has also been hypothesized for the sheet web spider *Florinda coccinea* (Roberston & Adler 1994) though it has not been tested directly. Interestingly, a male *Brachypelma klaasi*, a tarantula, has been observed behaving similarly where he deposited silk atop a female's silk surrounding her burrow without reducing the web. A second male was unable to locate the female's burrow afterwards (Yáñez et al. 1999). The purpose of the silk laying behavior has not been tested directly and the study only included three observations of the endangered species (Yáñez et al. 1999, Yáñez & Floater 2000).

Another form of manipulation occurs when a male induces a passive state in the female (termed here as quiescence) that allows the male to avoid being attacked and cannibalized (*Rabidosa*: Rovner, 1971.; *Agelenopsis*: Becker et. al. 2005; *Hololena*: Xiao et. al. 2015). Using the funnel weaving spider *Agelenopsis aperta*, Becker et al. (2005) investigated the importance of various aspects of the male's courtship in inducing quiescence in females using several isolating arenas. They found that males use a volatile semiochemical to induce females into a quiescent state and can do so effectively from a distance up to 3 cm. All females became quiescent within 0.5 cm of the courting male. Becker et al. (2005) were unable to determine the source of the male-produced chemical. However, their data suggest that the drumming performed by the male with his pedipalps during courtship is important for directing the chemical toward the female.

Spiders rely on chemical senses in multiple contexts (Uhl 2013) such as predator avoidance (Persons et al. 2002), prey localization (Hostettler & Nentwig 2006), habitat and foraging site selection (Bonte 2013, Heiling et al. 2004), and to locate and recognize conspecifics, especially in mating contexts (Tietjen & Rovner 1980). Spiders are covered with hairs that sense semiochemicals, like pheromones, with modified hairs that cover their legs and pedipalps (Tichy 2001, Ganske & Uhl 2018). These hairs have chemo-sensitive dendrites at the pore in the hair tip that can detect minute concentrations of pheromone (Tichy 2001). This type of tip-pore sensillum is the only confirmed chemoreceptor in spiders.

Male *Hololena curta*, another funnel weaving spider, show a similar ability to stun (or induce quiescence in) females but, unlike *A. aperta* males, they require direct physical contact with the female (Xiao et. al. 2015). In a series of mating trials, the authors tested the risk of female attack during individual components of male courtship. They concluded that the vibrational components of courtship – like those found in wolf spider courtship – are used by the male to reduce female aggression prior to mating. Inducing the quiescent state minimized attack risk during and post copulation. However, it is not known whether tactile stimulation or semiochemical deposition to the female cuticle is the cause of quiescence. Females of all successful copulations became quiescent once the male grasped her. Duration of the quiescent state post-copulation is unknown, but the female did become active shortly after the male released her from his grasp.

Scenarios such as that of *H. curta,* where behaviors have been documented but the mechanism involved is unknown, require a more careful examination of the animal to determine if they could be producing a semiochemical. One way to identify the source of chemical production in spiders is to search for cuticular structures on their legs and body with a scanning electron microscope. Scanning electron microscope (SEM) studies have led to the discoveries of chemoreceptors in many insects and arachnids (e.g., Coleoptera: Romero-López et al. 2004, Arachnida: Foelix et al. 1975) A few studies using SEM for cuticular structures and transmission election microscopy (TEM) for viewing the related ultrastructure, i.e., the tissues below the cuticle, have been conducted. SEM and TEM studies have not been conducted with many spiders, but

those that have been done show structures similar to those of insects (Noirot & Quennedey 1974, Kronestedt 1986, Tichy et al. 2001, Pekár & Šobotník 2007, Ganske & Uhl 2018). Unfortunately, studies like these have not been conducted for receptors on many animals and semiochemical production organ studies are even less common.

At maturation, the males of *Alopecosa cuneata*, a wolf spider, develop modified tibia on their first leg pair which become swollen and sclerotized (Kronestedt 1986). Females must grasp this region of the male tibiae during courtship for copulation to occur. Kronestedt (1986) found the sclerotized regions of the male tibiae to have an abundance of pits on raised, oblong structures. The ontological development of the pits and tibiae at maturation and the female requirement to grasp the male tibiae led Kronestedt (1986) to postulate that the pits emitted a pheromone. This was later supported by the finding that the pits were indeed connected to exocrine glandular cells via a canaliculus, a small duct (Juberthie-Jupeau et al. 1990). The function and product of the organs have not yet been identified.

Similar cuticular structures to those in *A. cuneata* have been observed on the legs of other genera of wolf spiders (Family Lycosidae, Kronestedt 1986) as well as in the ant spiders (Family Zodariidae, Pekár & Šobotník 2007). The pits of zodariid spiders are located on the femurs of their legs and covered by modified hairs making them less conspicuous compared to those of the Lycosids. Using TEM, Pekár & Šobotník (2007) show presumptive chemical producing glands and associated canaliculi but were not able to identify a product of the organ for any of the species with GC-MS. Behavioral evidence for the use of any putative semiochemical is also lacking though it is suggested

that one of the spiders studied, *Zodarion frenatum*, can paralyze their ant prey by contacting the ant with their legs (Harkness 1976 referenced by Jocqué & Dippenaar-Schoeman 1992).

In two species of a closely related genus in Zodariidae, *Diores termitophagus* and *D. magicus*, there have been observations of the same unique foraging strategy to *Z. frenatum*. These *Diores* spiders are termite specialists and do not bite their prey like most spiders but merely have to brush their termite prey with their legs to incapacitate it before consuming it (Jocqué & Dippenaar-Schoeman 1992). These two spiders as well as other *Diores* spiders have very similar femoral organs to the *Zodarion* species (Jocqué & Dippenaar-Schoeman 1992, Russell-Smith & Jocqué 2015) suggesting that the femoral organ has a role in their foraging behavior. Neither the foraging behavior nor the product of the gland have been determined yet. The zodariid spider examples above, obviously, are not in mating context. They do, however, provide valuable evidence of spiders possibly using a semiochemical produced from organs located on their legs to induce a subdued, or quiescent, state in another animal without biting it.

A second source to consider for male induced female quiescence is the male venom. Usually when a spider attacks and bites, it aims to subdue and kill a prey item. However, venom is energetically expensive and the reserves of it are small. Venom being so expensive and in low quantity has led to the evolution of very toxic and potent venoms that the spider can regulate the release of by flexing muscles associated with the venom gland (Peterson 2006). The components in venoms can be different among species, between the sexes within a species, and even within an individual over its

lifetime (Casewell et al. 2013) as the venoms have evolved with the animal for specific purposes like protection from predators and prey immobilization (Pekár et al. 2008, Casewell et al. 2013). Even though venom composition and use differ among animals, the overall components of venom are similar. That is, venoms are mostly proteins in a solution of salts, amino acids, and neurotransmitters (Casewell et al. 2013). Aggressive behaviors during mating have likely selected some species to bite or sting their mate during copulation (*Schizocosa ocreata*: Johns et al. 2009, scorpions: Sentenská et al. 2017a). Unfortunately, whether venom is transferred from the male to the female during these bites and stings is unknown, but some authors suggest the behavior may lower female aggression before copulation.

One other example of possible venom use during copulation has been proposed based on comparisons of venom chemistry and behavior of different species of long-jawed orb weavers (Family Tetragnathidae). Binford et al. (2016) analyzed the venom components of both wandering and orb-weaving tetragnathids with the expectation of finding larger differences in venom composition between the sexes of orb-weaving tetragnathids than the wandering species. They expected these differences in venoms based on the differences in feeding behaviors and ecologies of the species and sexes. Unexpectedly, they found large chemical composition differences between the sexes of almost all tetragnathid species tested without correlation to feeding ecologies. The males tended to have high concentrations of high molecular weight components and low concentrations of low molecular weight components while females displayed the reverse. This pattern is unlike any reported previously and the regularity among the

species brought Binford and colleagues to conclude that the males may be using their venom during copulation. However, this has not been tested.

A third possible source that may be used to manipulate females is the male produced ejaculate. In sexually reproducing species, males transfer ejaculate, containing sperm and seminal fluid, to female mates to fertilize her eggs and produce offspring. In the fruit fly *Drosophila melanogaster*, males produce a variety of accessory gland proteins (Acps) in their seminal fluid that are hypothesized to significantly reduce female receptivity to additional matings and reduce the life span of females in a dose dependent manner (Chapman et al. 1995, reviewed in Chapman & Davies 2004). These Acps peptides, a group of protease inhibitors, have multiple functions without being detrimental to the female while housed in her sperm storage organs, the spermathecae. However, these will also migrate to the female's hemolymph where they reduce the life span of the female. The peptide Acp62F, was considered the best candidate for the cause of the reaction but genetic deletion experiments show no differences in life-span post-mating when females mated with Acp62F deleted males (Mueller et al. 2008). The exact protein or group of proteins have not yet been determined in this reaction.

In spiders, the seminal fluid compounds and structures (Michalik 2009) within the fluid are highly diverse. In fact, one study (Michalik 2009) suggests every species of spider has unique secretions in the seminal fluids and that an individual species can have multiple secretion types present. While the biochemical composition of spider seminal fluids is unknown (Michalik & Ramírez 2014) the different secretions may have different purposes. These purposes range from forming spermatozoa sheaths to

nutrition for the spermatozoa while they are stored in the male pedipalps and female spermathecae, before being used to fertilize her eggs (Michalik 2009, Michalik & Ramírez 2014). The only behavioral evidence for male seminal fluids manipulating females in spiders is that of *S. malitiosa* where females become significantly less receptive in correlation with the number of insertions performed by the male (Aisenberg & Costa 2005, Estramil & Costa 2007).

Given the diverse strategies for males to manipulate females during copulation, in this study, I sought to determine the mechanism which male *Rabidosa rabida* wolf spiders use to induce quiescence in their female mates. To do this, I focused on three possible sources of chemical compounds that may be responsible for the quiescent state: pheromones produced from the male cuticle, venom, and ejaculate. These three focal sources were included in the study because of the background they all have in possible manipulation of mating partners.

Rabidosa rabida is a locally abundant wolf spider around Murray KY, USA and are found in grasslands and occasionally open woodlands. Specifically, they are found in the upper stratum of tall grasses in fields and lower herbaceous vegetation in open woodlands (Brady & McKinley 1994). During the mating season, male *R. rabida* find females by following their silk draglines that are laden with pheromones (Tietjen 1978, Tietjen & Rovner 1980). They will then court females with a series of leg extensions and pedipalp drumming/stridulation, described extensively by Rovner (1967, 1968, 1971, 1972). During copulation, the mating pair is positioned so the male is mounted on top of and antiparallel (i.e. facing the opposite direction) to the female (position II, Gerhardt

1924). From this position the male reaches between the female's fourth leg and abdomen to access her epigynum (a female spider's reproductive opening) with his pedipalp and haematodochal bulb (the male secondary sex organ) to make an insertion and transfer his ejaculate. While males of some spider species have mating systems where they only make single insertions with females, *R. rabida* males make multiple insertions with a single female without dismounting (Rovner 1971, 1972, Rovner & Wright 1975). During copulation, the female rotates her abdomen to allow the male access to her epigynum as he moves from side to side making insertions. Important to this study, the male regularly palpates the posterior-dorsal region of the female's cephalothorax, anterior-dorsal region of the abdomen and the dorsolateral regions nearby with his pedipalps and legs between insertion attempts.

Female *R. rabida* aggression has only been considered in experiments regarding the mating decisions and mating system of these spiders and not considered directly. Few comments have been made on their aggressive behaviors, but they are moderately cannibalistic. Male *R. rabida* appear to be at higher risk of being cannibalized pre-copulation, ~20% (Wilgers & Hebets 2012), as opposed to ~8% post-copulation (Rovner 1972). I could not find information on female attack rates for pre- or post-copulation aggression in *R. rabida*.

Male *R. rabida* are hypothesized to avoid cannibalism by stunning their female partner during copulation (Rovner 1971). Rovner (1971, 1972) briefly describes females in the quiescent state when males dismounted after mating and the females remained motionless. Rovner (1971) gently prodded some of the female's carapaces near their

abdomens with a thin paint brush handle and the females rotated their abdomens as if the males were still mounted and mating with them. Unfortunately, the frequency with which this behavior was observed was not reported though the motionless state lasted for six minutes in one instance (Rovner 1972). With these examples in mind, it appears the quiescent state is variable from species to species with regard to when the female is subdued or revived during courtship and copulation, the duration of quiescence, and the method of male induction of quiescence.

The overall goal of this study was to determine the mechanism behind male-induced female quiescence in the wolf spider *Rabidosa rabida* by investigating the compound responsible. Specifically, my objectives were as follows:

> **Objective 1 (Scanning Electron Microscope):** Investigate whether *R. rabida* wolf spiders have cuticular features that resemble those of insects and other spiders that are associated with known or presumed semiochemical production.
>
> **Objective 2 (Mating Trials):** Determine the source of semiochemical synthesis by the male.
>
> **Objective 3 (Homogenate Trials):** Examine whether physical contact is required for semiochemical transmission to the female, or whether it is volatile.
>
> **Objective 4 (GC-MS):** Describe the female quiescence inducing compound in question.

METHODS

Collection and Maintenance

Female and male *Rabidosa rabida* were collected from privately owned pastures near Murray, Kentucky, USA from early April to mid-June 2019 and early May to early June 2020 between sundown and midnight. Most individual spiders were collected as

juveniles. All spiders were housed in 5.8 × 5.8 × 7.6 cm plastic containers (AMAC Plastic Products Corp.,

Sausalito, CA) and kept on a 12:12 hour light cycle. Curtains were used to block any natural light that entered through the large windows of the housing rooms. Spiders were fed two ~12mm crickets twice a week during the 2019 season and two ~10mm crickets twice a week during the 2020 season. Water was provided *ad libitum* via cotton wicks inserted through the bottom of the plastic cages and partially submerged in water below the cages. While most individuals consumed the crickets provided in 2019, enough of the crickets were left incompletely consumed to lead to the decision to use smaller crickets in the 2020 season. All the crickets provided during feeding were completely consumed during the 2020 season. If crickets remained alive in the cages from a previous feeding day the feeding was modified so no more than two crickets were present in the cage at once – e.g. if one cricket remained in the cage from a previous feeding day, only one cricket was inserted. All individuals were monitored daily for molts to determine the date of maturation. Spiders were determined as mature by confirming the proper morphology of female epigynum and male pedipalps and coloration (Brady & McKinley 1994).

Objective 1 (Scanning Electron Microscope):

The Scanning Electron Microscope (SEM) was used to determine whether *R. rabida* wolf spiders had cuticular features that resembled those of insects and other spiders that are associated with known or presumed semiochemical production. Three spiders were processed and observed in the SEM at Hancock Biological Station at

Murray State University in Murray, Kentucky. One of each of the following spiders were used: juvenile male, mature female, and mature male. Juvenile females were not included due to the difficulty of properly assessing the sex and penultimate instar of juvenile spiders. Juvenile male spiders have swollen, developing pedipalps at their penultimate instar making it easy to identify them while juvenile females do not. This developmental difference meant I could incorrectly select a juvenile male instead of a juvenile female so juvenile females were not considered.

All the spiders were euthanized by freezing before being put through a dehydration series. The dehydration series consisted of three steps of deionized water, 50%, 75%, and 95% ethanol solutions, three steps of 100% ethanol, one step of solution made of 100% ethanol and acetone in a 1:1 ratio, and one step of 100% acetone. The spiders were set in the first six steps for five minutes each, the three ethanol steps for ten minutes each, and the final two steps for fifteen minutes each. After the dehydration series, the specimens were then dried in a Denton Vacuum DCP-1 critical point drying apparatus with acetone and carbon dioxide. The dried spiders' legs, pedipalps, cephalothorax and opisthosoma were then dissected and mounted on steel stubs with carbon tape or, if needed, silver paint (Ted Pella Inc., Redding, CA) and coated with a thin layer of gold (~0.2nm) over two minutes in an Anatech LTD Hummer VI sputtering system. The samples were observed in a JEOL Scanning Electron Microscope with an accelerating voltage of 5kV.

Objective 2 (Mating Trials):

Mating trials were conducted in the laboratory with manipulated males – males with ablated body regions – to determine possible sources of semiochemical production leading to female quiescence. A total of 113 mating trials were conducted (73 in 2019 and 40 in 2020) to determine the mechanism used by *R. rabida* males to induce the quiescent state in females. The 73 trials performed in 2019 included females that ranged in age from 15 to 74 days post maturation and males that ranged from 14 to 44 days post maturation. Results of the 2019 trials caused me to decide that females at ≥30 days post maturation did not yield reliable data. The preliminary results suggested older, non-virgin females behaved differently than younger females and, therefore, were not reliable for the current study and were excluded from the final analysis (see Results, Objective 2). The 40 trials performed in 2020 included females that ranged in age from 12 to 21 days post maturation and males of ages 14 to 28 days post maturation. All trials were performed with known virgin females and known virgin males that matured in the laboratory.

All mating trials were conducted in round 9 cm (h) x 26 cm (d) plastic arenas (250C, Pioneer Plastics, North Dixon, KY) with filter paper on the arena floor. The arenas sat on a 30 cm x 30 cm granite tile on the laboratory bench. The arenas were cleaned with seventy-five percent ethanol solution between trials and new filter paper was used for all trials. All trials were live-scored and video-recorded (camcorder: Sony Handycam HDR-PJ540). All 2019 trials were conducted from 8 June to 15 August between 0750 and 1620 hours. All 2020 trials were conducted from 14 June to 2 July between 0820 and

1340 hours. Each female-male trial pair was randomly selected using filtering and randomizing functions in Google sheets.

Male *R. rabida* were randomly selected and randomly categorized into a control group or one of four treatment groups (ablation control, ablated fangs, ablated pedipalps, ablated legs). Males in the treatment groups were ablated the day prior to their mating trial to allow the ablation treatment time to dry and the male time to acclimate to the ablation. Female *R. rabida* were randomly selected using the same methods as for the males. They were introduced to the mating trial arena the day prior to their trial to allow them time to deposit pheromone laden silk (Tietjen 1978, Tietjen & Rovner 1980) in the arena and become acclimated to the arena itself. A small water-soaked cotton wick in a small vial cap was provided to avoid dehydration overnight. The wick and cap were removed immediately before the start of the trial. Two crickets were also provided at the beginning of the acclimation period to ensure any aggression by the female was not caused by hunger. Any remaining crickets were removed immediately before the mating trial. Males were introduced to the arena with the female at the furthest point away from the female. The trial was concluded if the pair had not begun copulation after 30 minutes. For pairs that mated, a small plastic cup was set over the male after he dismounted the female and moved away. The trial was concluded when the female became active after being quiescent post-copulation. The quiescent state was determined by the regular mating stance – the female with her sternum near the ground, abdomen in the air, and legs extended straight on the ground – versus her active standing position body raised above the ground.

To ablate the male body parts, the males were first cold anesthetized for three minutes at -20°C. This was done to allow for easier handling of the spider during the following steps. After anesthetization they were inserted into a plastic sandwich bag and positioned into a sprawled posture so none of their legs were underneath them. Ablation control and ablated leg males were positioned dorsal side up. Ablated fangs and ablated pedipalp males were positioned ventral side up. Sewing pins were then used to secure the spider to a cube of packing Styrofoam. The plastic bag was cut with a razor blade where needed for the ablation and more pins used to keep the bag from obstructing the ablation process. All ablations were completed with superglue (Krazy Glue, Elmer's Products Inc.) under a dissection microscope. Males of the ablation control group received a drop of glue on their carapace (**Figure 1a**). Male's legs were ablated by applying glue to all sides of the femur, patella, tibia, and metatarsus of the first two leg pairs with care to avoid gluing joints (**Figure 1b**). The pedipalps were ablated by applying a drop of glue to the emboli and haematodochal sacs of their pedipalps (**Figure 1c**). Lastly, the fangs were gently teased out from the cheliceral furrow with a teasing needle, glue applied to the fang, and the fang returned to the cheliceral furrow (**Figure 1d**). Occasionally the glue dried as the spider flexed its fangs leaving the fang in a partially exposed position.

Objective 2 (Mating Trials): Statistical Analyses

Female Age 2019 (trials leading to smaller age range)

Preliminary analyses were conducted after the 2019 trials were finished. Rates of mating pairs and quiescent females were calculated separately. The probability of the female becoming quiescent among treatments was determined by creating a

contingency table, using the 'table()' function, with the female quiescent state (yes/no) and treatment. The table was then used to perform a Fisher exact test with the 'fisher.test()' function in R version 4.0.3.

Several one-way ANOVAs were performed to determine the influence of the male ablation treatment on behavioral outcomes in the trials. These behavioral outcomes included latency to courtship, latency to copulation, duration of copulation, and duration of the female quiescent state. All 2019 trials were used in the analyses for latency to courtship, latency to copulation, and duration of copulation. Only trials with females that were quiescent post-copulation were used in the analysis testing differences in duration of the female quiescent state. These analyses were conducted in JMP 14 (SAS Institute Inc.).

The effect of female age, female weight, male age, male weight, and ablation treatment on the female's quiescent status (yes/no) post-copulation was analyzed with a nominal logistic model to eliminate confounding variables. The same predictors and trials were used in an ANOVA to determine whether they influenced the duration of female quiescence. The distribution of female age among treatments was calculated with an ANOVA to ensure no differences in female age affected the trial outcomes as was shown from the 2019 mating trials (see Results, 2019 Age and Weight Effects). These analyses were performed in JMP 14 (SAS Institute Inc.) with all 2019 trials.

Stats to ensure the spiders behaved similarly 2019 and 2020

Latency to and Duration of Courtship and Copulation

To ensure the ablation treatments did not affect the behavior of the male *R. rabida* I conducted multiple analyses with the 'anova()' function and the 'TukeyHSD()'

function, where applicable, in R version 4.0.3. I measured the time it took males to start courting females (latency to courtship), the duration of the male courtship, the time it took for copulation to start (latency to copulation), and the duration of copulation. Separate tests were performed to compare the trials from all treatments across both years as well as the trials from all treatments between years to check that there were no behavioral differences between the trial years.

Probability of Quiescence and Female Quiescence Durations

The probability of the female being quiescent post-copulation due to the male treatment was determined by creating a contingency table, using the 'table()' function, with the female quiescent state (yes/no) and treatment. The table was then used to perform a Fisher exact test with the 'fisher.test()' function. To determine which treatments were significantly different from each other the same table was used in the 'pairwise_fisher_test()' function. Further analyses were conducted to check for other predictors of female quiescence in a nominal logistic model. The 'glm()' function was used with 'family = binomial (link = logit)' to code for the nominal logistic regression. The significance of each predictor was then determined using the 'Anova()' function. These tests were conducted in R version 4.0.3. The predictor variables treatment, female weight, female age, male weight, and male age were used to test for their influence on whether the female became quiescent and to eliminate confounding variables. The durations of female quiescence among treatment groups were examined using the 'anova()' function in R version 4.0.3.

Pre-copulation and Post-copulation Attacks and Cannibalism

Statistical analyses were conducted to address female aggression pre- and post-copulation. Two separate Fisher exact tests were used to determine whether the treatment predicted the number of females that attacked their partner (yes/no) per treatment pre- and post-copulation. The pre-copulatory attack test included all trials (n = 80). The post-copulatory attack test included only the pairs that copulated (n = 58). The Fisher exact tests were performed with a contingency table (treatment by female attack occurrence (yes/no)) in the 'fisher.test()' function in R version 4.0.3.

Three separate tests were used to determine if mated, non-mated, quiescent, or non-quiescent females and females grouped by treatment were more likely to attack their male partners pre-copulation. First, an ANOVA was used to determine if female pre-copulatory attacks differed by treatment. This test used all trials, including trials where females did not attack the male. Next, an ANOVA test determined whether females of pairs that copulated attacked more pre-copulation than pairs that did not copulate. Whether the pair copulated (yes/no) was used as a predictor of number of pre-copulatory attacks by the female to check for differences in aggressive behavior between the two groups. All trials were considered in these analyses. Lastly, a t-test was used to determine whether females that became quiescent were more likely to attack post-copulation than females that were not quiescent post-copulation. All total pre-copulatory attacks included attacks that concluded in sexual cannibalism. The ANOVA analyses were performed with the 'anova()' function and the t-test with the 't.test()' function in R version 4.0.3.

Similar to the pre-copulatory attacks, separate tests were performed to determine if quiescent or non-quiescent females or females of certain treatments were more likely to attack their partners post-copulation. An ANOVA was performed to determine whether the male treatment influenced the number of female attacks using all trials (n = 80). Next, a t-test determined whether female's quiescent state (yes/no) post-copulation influenced the probability of post-copulatory attacks (n = 58). Another ANOVA was performed to test the influence of treatment type and whether the females had become quiescent (yes/no) on post-copulatory attacks (n = 58).. All total post-copulatory attacks include attacks that concluded in sexual cannibalism. The ANOVA analyses were performed with the 'anova()' function and the t-test with the 't.test()' function in R version 4.0.3.

Objective 3 (Homogenate Trials):

A total of 37 homogenate trials were conducted from 19 June 2020 to 13 July 2020 between 0958 and 1808 hours. Females ranged from 14 to 28 days old post maturation molt. Males ranged from 15 to 41 days old post maturation molt. Results from the 2019 mating trials suggested female age but not male age affected the trial outcome (see Results, 2019 Age and Weight Effects), thus allowing us to use a wider age range for males. Preliminary study trials using a homogenized male *R. rabida* in protein buffer presented to female *R. rabida* were conducted. This experiment aimed to determine whether the quiescent response of the female spiders was caused by direct contact or by the delivery of volatile chemicals.

The homogenate trials were conducted in a 17.5cm (l) x 10.7cm (w) x 10cm (h) plastic container (Lee's Kritter Keeper, San Marcos, CA) with clean filter paper placed on the floor. The arenas sat on a 30 cm x 30 cm granite tile on the laboratory bench. The arenas were cleaned with 75% ethanol solution between trials and new filter paper was used for all trials. All trials were live scored and video recorded (camcorder: Sony Handycam HDR-PJ540). Female spiders, male spiders, and treatment type were randomly selected with filtering and randomizing functions in Google sheets.

A buffer solution was used in all trial treatments except in a distilled deionized (DDI) water control. I used the same buffer solution as was used in a similar study with *Agelenopsis aperta*, a funnel weaving spider, by Becker et al. (2005). The buffer contained 0.014 g 5 mM Hepes, 2.4g 70 mM sucrose, 4.0g 220 mM mannitol, 0.2 ml 0.5M ethylenediamine tetra acetic acid (EDTA), 200 µl bovine serum albumin (BSA), and 100 ml of deionized (DI) water. The ingredients listed were all measured as listed except the EDTA which needed to be dissolved in DDI water prior to taking the proper aliquot. The EDTA solution was made by dissolving 18.61g EDTA into 50ml DDI water with sodium hydroxide to help the EDTA dissolve. After the EDTA dissolved, the solution was diluted with another 50ml DDI for a total volume of 100ml of solution. The final buffer was stabilized to 7.42pH, the physiological pH of most organisms, with sodium hydroxide or hydrochloric acid as needed.

Female *R. rabida* were randomly selected and placed into one of six treatment groups. These treatment groups were: 1) DDI water on abdomen control, 2) buffer on abdomen control, 3) male leg homogenate on abdomen, 4) male body homogenate on

abdomen, 5) buffer on floor control, and 6) male homogenate on floor. For the first four treatments, I used a #2-pointed artist's paint brush to apply the DDI water, buffer, or homogenate to the anterior dorsal side and left and right adjacent regions because these are the areas of the female abdomen where the male palpates and rubs his legs during copulation. Treatments 3 & 4 were designed to identify the location of semiochemical production by the male and if palpation was the mode of deposition to the female. Treatments 5 and 6 were designed to determine if the chemical was transferred by a volatile vapor. For these treatments, I deposited 1 ml of buffer or homogenate on the filter paper of the arena.

For all trials, the female was placed in the arena and given a ten-minute acclimation period prior to the start of the trial. For treatments 1-4, the trials lasted for ten minutes and consisted of regular applications of the appropriate solutions with the brush. Attempts to apply the solutions occurred approximately every five seconds. Some attempts did not contact the female spider due to her retreating from the brush – this was not quantified. If the female retreated or appeared aggressive towards the brush, she would be given a short amount of time to settle back to a regular standing position before attempting to apply the solution again. The brush was reloaded with solution regularly throughout the trial. This replenished the solution in the brush and kept the solution in the holding beaker from settling. Any excess solution was removed from the brush by touching it to the side of the small beaker after reloading and before attempting to apply it to the spider. For treatments 5 and 6, 1 ml of homogenate was

deposited in the center of the arena at the start of the trial and the spider could walk near and over it freely for 20 minutes.

For the trials that required a male homogenate (treatments 3, 4, and 6), a male was randomly selected just before the trial. The male was then anesthetized at -20^{o}C for ten minutes or until it was unresponsive but not frozen to death. The duration needed to cold anesthetize the males was determined before conducting the trials. Males were inserted into the freezer and monitored until they were unresponsive. Occasionally, a male required extra time in the freezer to become unresponsive. No males were exposed to the cold for more than 14 minutes. After cold anesthetization, the male was sacrificed by removing its abdomen from its cephalothorax by the cutting the pedicel. The legs were then also removed from the cephalothorax at the coxa – the most proximal leg section. After sacrificing the male and dissecting its legs, the body parts were weighed. The respective body parts were then ground in a mortar and pestle for one minute and the room temperature protein buffer added to the ground male in a 2ml:0.1g ratio (Singer & Reichert 1995, Becker et al. 2005) . For treatment 3, only the male's legs were homogenized. Similarly, only the male body – both cephalothorax and abdomen – were homogenized for treatment 4. The entire male was homogenized for the solution used in treatment 6.

Objective 3 (Homogenate Trials): Statistical Analyses

An ANOVA was used to explore whether homogenate treatment type determined the duration of the quiescent state. Only females that had become quiescent were used in this test. The 'anova()' function in R version 4.0.3 was used for

this analysis. A contingency table and Fisher exact test were used to determine if treatment type influenced the occurrence of female quiescence. The contingency table was built using quiescent state (yes/no) by treatment in the 'table()' function and tested with the 'fisher.test()' function in R version 4.0.3.

A nominal logistic regression was performed to determine predictors of female quiescence. The predictor variables included were female age, female weight, and treatment to eliminate confounding variables. The regression was conducted using the 'glm()' function with 'family = binomial (link = logit)' to build the model and the 'anova()' function to test it. These analyses were performed in R version 4.0.3.

Objective 4 (Gas Chromatography-Mass Spectrometry):

Preliminary chemical analyses were conducted to determine the chemical *R. rabida* males produce to induce conspecific females into the subdued quiescent state during copulation. In July 2020, during the regular mating season for *R. rabida*, I dissected four *R. rabida* and analyzed a variety of their body parts and organs with gas chromatography-mass spectrometry (GC-MS) to determine whether males produced quiescence-inducing chemicals. One juvenile female, one juvenile male, one mature female, and one mature male were used to compare the output chemicals from the GC-MS between the different sexes and maturities of the spiders. The samples collected and analyzed from the spiders included the cephalothorax and abdomen together, all eight legs, both venom glands, and the males' pedipalps. The juvenile female used was selected based on size and lack of swollen pedipalps because of the difficulty identifying the sex of a juvenile female spider as described in the methods for Objective 1.

Dissecting the spiders involved similar methods to those described for obtaining male homogenates. First, the spiders were cold anesthetized at -20^{o}C for 10 minutes and sacrificed by cutting the pedicel with a razor. The legs were then removed from the cephalothorax at the coxa with a razor. Venom gland removal was done similarly to Garb (2014) which required holding the remaining cephalothorax under a dissecting microscope with forceps, cutting the cuticle lateral to the chelicerae with a second set of forceps, and gently grasping the chelicerae and teasing the venom glands out. Once separated, as much of the remaining cuticle of the chelicerae was removed as possible without damaging the glands. Lastly, the pedipalps of the male spiders were removed. The pedipalps of the immature male were cut at the tibial-tarsal joint and required no further dissection. The mature male pedipalps were removed similarly with the addition of removing as much of the cymbium as possible, leaving only the haematodochal sac and embolus for analysis. All samples were immediately transferred to 2mL GC-MS glass vials, submerged in a 1:1 hexane and acetone solution, and crushed with hard forceps until no large pieces remained. Additionally, the venom gland and pedipalp samples required vial inserts (250µL, glass with polymer feet, Agilent, Santa Clara, CA) in the vials to allow the GC-MS to properly extract the sample. The samples were then placed in a bath sonicator (Branson Ultrasonic) set at 40 kHz for 30 minutes before analysis in the GC-MS. An additional 100 µL of the hexane-acetone solution was also needed for the venom gland and pedipalp samples for the machine to access the solution post sonication. The abundances of each compound per sample were not measured.

The vials with the processed samples were then inserted into an autosampler (Agilent Technologies 7693) and analyzed with an Agilent Technologies 7890A gas chromatograph coupled with an Agilent Technologies 5975C with Triple-Axis mass spectrometer detector. The GC was equipped with a 30 m x 250 µm x 0.25 µm film thickness column (HP-5MS Agilent Technologies). For all samples, 1 µL of the sample was injected at 300°C inlet temperature with spitless mode injection. The oven temperature was programmed at 50°C to start and held for 30 seconds. Ramp one started at 15°C for 1 minute and increased to 200°C for 1 minute. Ramp two started at 40°C for 1 minute and increased to 300°C. Total analysis time was 16 minutes per sample. The auxiliary heater was set to 250°C and the ion scan range was 50 – 450 *m/z*. Suspect peaks were massed with EI mist library (NIST08).

RESULTS

Objective 1 (Scanning Electron Microscope)

The three spiders used in the scanning electron microscope (juvenile male: **Figure 2a**, mature female: **Figure 2b**, mature male: **Figure 2c**) all had cuticular pits on the femur, patella, tibia, metatarsus, and tarsus of all legs. The structures varied slightly in size, and shape. In general, the entire structures were oblong and tapering from wide to narrow with an opening, a “pit”, on the narrow end. The entire structures were approximately 5µm long and 2 to 4µm wide. The pits were approximately 1µm in diameter or less. The structures and pits, in relation to each other, were not arranged in any apparent order. That is, they were not all oriented in the same direction along the leg segments and do not appear to be equally spaced.

Objective 2 (Mating Trials)

In the 2019 mating trials (n = 73), including the females older than 30 days post maturation that were later excluded from the combined analyses, 53-73% of the pairs mated . Sixty-seven percent of the control (n = 10/15), 69% of the ablated control (n = 9/13), 60% of the ablated fangs (n = 9/15), 73% of the ablated legs (n = 11/15), and 53% of the ablated pedipalps (n = 8/15) pairs mated. Of the pairs that mated in the 2019 trials (n = 47), between 18-67% of females became quiescent and remained in the state post-copulation. Forty percent of the control (n = 4/10), 67% of the ablated control (n = 6/9), 67% of the ablated fangs (n = 6/9), 18% of the ablated legs (n = 2/11), and 63% of the ablated pedipalps (n = 5/8) females were quiescent post-copulation. There was no significant difference in rates of females in quiescence among treatments (Fisher exact test: p = 0.124).

The initial preliminary behavioral analyses (2019 only, n = 73) indicate that the male ablation treatments did not affect the courtship and copulation behaviors. There were no significant behavioral differences in latency to courtship (ANOVA: $F_{4,68} = 0.914$, p = 0.461) among treatments. On average, males took 127 seconds in control (n = 15), 194 seconds in ablation control (n = 13), 77 seconds in abated fangs (n = 14), 228 seconds in ablated legs (n = 13), and 250 seconds in ablated pedipalps (n = 13) to begin courting. There were no significant behavioral differences in latency to copulation (ANOVA: $F_{4,68} = 1.032$, p = 0.397) among treatments. On average, males took 585 seconds in control (n = 10), 509 seconds in ablated control (n = 9), 613 seconds in ablated fangs (n = 9), 884 seconds in ablated legs (n = 11), and 718 seconds in ablated

pedipalps (n = 18) to begin copulation. There were no significant behavioral differences in copulation duration (ANOVA: $F_{4,68} = 1.368$, $p = 0.254$) among treatments. On average, pairs mated for 6343 seconds in control, (n = 10), 4594 seconds in ablated control (n = 9), 8118 in ablated fangs (n = 9), 5652 seconds in ablated legs (n = 11), and 3408 seconds in ablated pedipalps. Lastly, all females that were quiescent post-copulation (n = 23) were quiescent for similar durations among treatments (ANOVA: $F_{4,18} = 0.699$, $p = 0.603$). Females were quiescent, on average, for 223 seconds in control (n = 4), 95 seconds in ablated control (n = 6), 73 seconds in ablated fangs (n = 6), 68 seconds in ablated legs (n = 2), and 141 seconds in ablated pedipalps (n = 5).

2019 Age and Weight Effects

In the 2019 mating trials, female age ranged from 15 to 74 days post maturation with a median of 27 days (n = 73). In the same trials the males ranged from 14 to 44 days old post maturation with a median of 24 days (n = 73). In these trials (n = 73) only female age was a predictive factor for the occurrence of the quiescent state (Nominal Logistic Regression: $X^2 = 15.256$, $df = 1$, $p < 0.0001$). Specifically, older females were less likely to become quiescent and no females older than 35 days post maturation became quiescent (**Figure 3**). Female weight ($X^2 = 0.028$, $df = 1$, $p = 0.867$), male age ($X^2 = 2.248$, $df = 1$, $p = 0.134$), male weight ($X^2 = 0.588$, $df = 1$, $p = 0.443$), and treatment ($X^2 = 6.556$, $df = 4$, $p = 0.161$) were not predictive of the quiescent state in the same model. Furthermore, the duration of female quiescence was not influenced by any of these factors either (ANOVA: female age: $F = 1.539$, $df = 1$, $p = 0.219$; female weight: $F = 0.363$, $df = 1$, $p = 0.549$; male age: $F = 0.065$, $df = 1$, $p = 0.800$; male weight: $F = 0.294$, $df = 1$, p

= 0.589; treatment: F = 0.350, df = 4, p = 0.8429). Female ages were equally distributed across the treatments in these trials (ANOVA: F_4 = 0.440, p = 0.779).

The analyses from the 2019 trials above showed trends leading us to make minor changes to the methods for the 2020 mating trials with ablated males. Specifically, I narrowed the age range of females to better reflect the age which they would mate *in situ*. The female age range used for the remaining statistics was 12 to 28 days post maturation.

Latency to and Duration of Courtship and Copulation

The males all courted and copulated normally both years the trials were conducted which suggests that the treatments did not affect their ability to recognize the female pheromones, court, or copulate in the mating trials. The male age range was not a significant factor for this study, so I did not limit the age of males' post maturation in the 2020 mating or homogenate trials.

Ninety-five percent of males courted (n = 76/80). The remaining four males were in the ablated pedipalps (n = 2), ablated legs (n = 1), and ablated fangs (n = 1) treatment groups. Males took approximately the same amount of time to begin courtship in all treatments across both years (ANOVA: $F_{4,71}$ = 0.572, p = 0.683, **Figure 4**) and between years (ANOVA: $F_{9,66}$ = 1.243, p = 0.285). Males also courted for the same amount of time among treatments (ANOVA: $F_{4,53}$ = 0.732, p = 0.575, **Figure 5**) and between years (ANOVA: $F_{9,48}$ = 0.736, p = 0.674). Seventy-six percent of the males that courted also started copulating (n = 58/76) in the first thirty minutes of the trial.

Seventy-three percent of the pairs mated overall (n = 58/80) and most of the pairs in each treatment mated (65 to 84%, **Figure 6**). Pairs that mated began copulation the same amount of time after the trial start among treatments (ANOVA: $F_{4,53} = 0.931$, p = 0.453), and between years (ANOVA: $F_{9,48} = 0.720$, p = 0.688).

Copulation durations were statistically different among treatment groups (ANOVA: $F_{4,53} = 2.656$, p = 0.043) but only between pairs with ablated pedipalp males and ablated fangs males (TukeyHSD: p adjusted = 0.037). The ablated fangs males had longer copulation durations among the treatments and the ablated pedipalp males had the shortest (**Figure 7**). However, when year was considered, there was no significant difference among the groups anymore (ANOVA: $F_{9,48} = 1.406$, p = 0.212). This may indicate that this is not a robust pattern since the two analyses do not agree whether or not the difference in copulation duration is significant.

Probability and Duration of Female Quiescence

The probability of the female entering the quiescent state was predicted by the male treatment (Fisher exact test: p = 0.008) but only between ablated fangs males and ablated legs males (pairwise Fisher exact test: p-adj. = 0.010, **Figure 8**). Females paired with males of the ablated legs treatment were the least likely to be quiescent at the end of copulation (n = 3/11 or 27%, **Figure 8**) and the other four treatments ranged from 56-100%. Treatment (Nominal Logistic Regression: $X^2 = 14.648$, df = 4, p = 0.005) was the predictor of female quiescence again when tested against female weight ($X^2 = 0.661$, df = 1, p = 0.416), female age ($X^2 = 0.038$, df = 1, p = 0.845), male weight ($X^2 = 0.000$, df = 1, p = 0.997), and male age ($X^2 = 0.200$, df = 1, p = 0.655).

Of the females that became quiescent, all were in the state the same amount of time among treatments (ANOVA: $F_{4,32} = 0.816$, $p = 0.524$, **Figure 9**). Females were quiescent for 131 sec in control, 93 sec in ablation control, 51 sec in ablated fangs, 125 sec in ablated legs, and 171 sec in ablated pedipalps treatments on average with no significant difference among them.

Pre-copulation and Post-copulation Attacks and Cannibalism

During the mating trials, 20-53% of females attacked their mates prior to copulation (n = 80) and 10-55% of females attacked their mates post copulation (n = 58, **Figure 10**). The number of pre-copulatory attacking females did not differ among treatments (Fisher exact test: $p = 0.424$). Post-copulatory attacking females paired with leg ablated males were at least twice as likely to attack males compared to females of other treatments. Although, statistically, the number of post-copulatory attacking females did not differ significantly among any of the treatments (Fisher exact test: $p = 0.262$).

Two cannibalisms of the male by the female occurred during the mating trials pre-copulation (n = 80, 2.5%) and 5 cannibalisms occurred post-copulation (n = 58, 8.6%). The total number of cannibalisms per treatment are similar among treatments (**Table 1**).

Pre-copulatory attacking females attacked their partners the same amount among treatments (ANOVA: $F_{4,75} = 0.453$, $p = 0.769$) but females of pairs that did not mate attacked more often (ANOVA: $F_{1,78} = 7.827$, $p = 0.006$). Females attacked males

just as often prior to copulation whether or not they became quiescent post-copulation (t-test: $t_{56} = 0.568$, $p = 0.572$) (**Figure 11**).

Post-copulatory attacking females attacked their partners the same amount among treatments (ANOVA: $F_{4,75} = 0.675$, $p = 0.612$). Female quiescent state did influence the number of post-copulatory attacks by the female. Specifically, females in the quiescent state attack the males significantly less often post-copulation (t-test: $t_{56} = 3.600$, $p < 0.001$, **Figure 11**) but this was not influenced by treatment (ANOVA: $F_4 = 0.877$, $p = 0.484$).

Objective 3 (Homogenate Trials):

In the homogenate trials, females of three treatments that had male homogenate or buffer applied directly to them (treatments 2, 3, and 4) became quiescent (**Figure 12**). The females that did become quiescent (n = 9) were in the state for the same amount of time in each treatment (ANOVA: $F_{2,6} = 0.875$, $p = 0.464$). The females were in quiescence for an average of 224 seconds with the brush and buffer control, 511 seconds with the brush with body homogenate, and 519 seconds with the brush and leg homogenate (**Figure 13**). Treatment type did predict whether a female would become quiescent (Fisher exact test: $p = 0.012$; Nominal Logistic Regression: $X^2 = 16.931$, $df = 5$, $p = 0.005$) while female weight ($X^2 = 0.749$, $df = 1$, $p = 0.387$) and female age ($X^2 = 1.689$, $df = 1$, $p = 0.194$) did not.

Objective 4 (Chemical Analysis):

The highest-ranking library matches from the GC-MS were selected after the analysis and summarized in **Table 2**. Overall, I identified these fifteen classes of

compounds among all the samples used in the GC-MS analysis: 6 alkanes, 2 alkenes, 1 alkylbenzenes, 1 alkyne, 1 butyl ester, 1 cholesterol, 1 cholesterylene, 1 ether, 1 ethanolamine, 4 fatty acids, 1 fatty acid amide, 2 hormones, 1 non-proteinogenic amino acid, 1 oxime, 2 piperidines, 1 polyunsaturated hydrocarbon, 1 steroid, 1 substituted alcohol, and 1 substituted phenol .

The compound that occurred the most often across samples was 1,3-bis(1,1-dimethylethyl)-benzene, a volatile alkylbenzene (PubChem, Chemspider). This compound was found in nine of the fourteen samples. This was the only compound returned from the mature female legs, and notably, the mature male leg samples. The second most frequently occurring compound was 2,4-bis-(1,1-dimethylethyl)-phenol, an alkylbenzene with antibacterial, antifungal, and antioxidant activities, as well as being used as repellents and deterrents (reviewed in Zhao et al, 2020). This compound was found in various samples from the juvenile female, juvenile male, and mature female. It was not detected in the mature male. The third most abundant compound was N,N-dimethyl-2-aminoethanol (Deanol), an ethanolamine that reacts with and eliminates radicals (PubChem, Chemspider). Deanol was found only in juvenile samples. All the remaining compounds were found in three or fewer of the samples.

DISCUSSION

The present study examined how *Rabidosa rabida* males induce a quiescent state in females during copulation. The results of 4 experiments reveal that more than one mechanism is likely involved and that these mechanisms may interact to subdue the female. With the first objective, I discovered pits on the cuticle of the walking legs of

adult and juvenile spiders, suggesting not only that chemicals could be released from these pits during pair formation, and that chemical communication may also be an important sensory modality throughout life. Next, I conducted an experiment using mating trials that selectively blocked potential chemical-emitting sources on the male (fangs, pedipalps, and forelegs) and quantified whether the female became quiescent during female-male interactions. This experiment revealed that when females were paired with males that had the cuticular pits on the legs ablated, they were less likely to become quiescent. This result strongly suggests that males are transferring a chemical to subdue the female from the pits on their walking legs. I also tested whether quiescence could be induced in the absence of a live male by presenting homogenate solutions of localized regions of a sacrificed male to the female either by direct contact or as a volatile solution. This homogenate experiment revealed that tactile pressure may play a role in inducing the quiescent state. Finally, in a preliminary study, I attempted to identify the putative chemical compound(s) used by the male to subdue the female. Although the chemical analyses of the venom glands, ejaculate, and legs did not provide conclusive evidence. A more in-depth investigation of the components could reveal a putative sedative-like chemical.

Objective 1

The pits that were found on the spiders' legs via SEM, which may have been more numerous in mature males (though not quantified), are presumed to be associated with semiochemical-producing organs and not chemosensory pits because spiders are known to have chemosensory hairs used for gustatory and olfaction (Tichy et

al. 2001, Ganske & Uhl 2018). Additionally, the tarsal organ is known for sensing humidity and temperature (Ehn & Tichy 1994) and are thought to have olfactory capabilities as well (Tichy & Loftus 1996). The lack of known pheromone-emitting structures, behavioral evidence for pheromone production, and similarity to pheromone-emitting structures of insects (Noirot & Quennedey 1974) suggests to me that these structures are used for semiochemical production. Additional behavioral, SEM, and TEM studies together with the behavioral results from the current study imply these pits are pheromone-emitting organs (Kronestedt 1986, Jocqué & Dippenaar-Schoeman 1992, Pekár & Šobotník 2007). That the cuticular structures were found on all the legs of both sexes and both mature and immature individuals suggest they are needed throughout the spider's life, and not just for mating and cannibalism avoidance contexts.

Objective 2

The lower likelihood of quiescence from pairing with leg-ablated males as found in the mating trials (objective 2; **Figure 8**) provide further support that the legs are the likely location of potential pheromone-emitting cuticular structures. However, since there was only a significant difference between the leg and fang treatments, and no other pairwise differences, further study is needed with larger sample sizes to confirm these findings. The mating trials also provided support that quiescence serves to prevent attack, since non-quiescent females were more likely to attack (**Figure 11**).

Evidence for semiochemical production and the organs from which they originate in spiders is mixed or lacking altogether. In the desert agelenid *A. aperta*,

behavioral evidence shows males use a volatile pheromone to induce females into a quiescent state without the need to touch her (Becker et al. 2005). Unfortunately, no work has been conducted to determine the source of the pheromone in *A. aperta* but work with other species suggests pits on the males' legs (*A. cuneata*, Kronestedt 1986). There is also evidence of a predatory semiochemical used by *Zodarion* spiders. This is likely emitted from similar structures which are associated with glands below the cuticle (Jocqué & Dippenaar-Schoeman 1992, Pekár & Šobotník 2007). In spiders, cuticular compounds can be used for intraspecific communication and recognition (Trabalon 2013), which suggests use of any produced semiochemicals for simple communication between a female-male pair in courtship and copulation contexts. In this scenario, the female, recognizing the male by his cuticular compounds, and presumably having mature eggs (she is physiologically going through vitellogenesis) may simply be receptive to the male and accept him as a mate. However, my data suggest the cuticular structures play a role in inducing the females into the quiescent state rather than just for conspecific recognition.

Since the mating pairs in all the treatments behaved similarly during courtship and copulation (**Figures 4, 5, 6**), with the exception of copulation duration between ablated pedipalps and ablated fangs males (**Figure 7**; discussed below), they did not appear to be otherwise affected by the ablation process. If cuticular compounds needed to be exchanged for conspecific recognition, they would have been able to do so as the leg ablation was performed on four (out of five) leg segments on the first two pairs of legs and the rest of the male was left unmodified. Additionally, the likelihood of

quiescence between females paired with leg- and fang-ablated males being significantly different suggests a product transferred from the males' legs is used to induce quiescence and not just to initiate mating (**Figure 8**). If the cuticular structures were used only for mate recognition, I would have expected fewer females to recognize males and mate, but this was not the case.

I observed mating occur at similar rates among all the treatments but as previously mentioned, females paired with leg-ablated males became quiescent less often. The result that females were quiescent for similar durations across all treatments (**Figure 9**) suggests the males need to use their cuticular organs to induce the state with little or no effect of dosage. If there were an effect of dosage the expected outcome would be that the duration of quiescence would have also been shortest in the leg ablation treatment simply due to nearly half of the total number of cuticular structures on the legs being unable to transfer pheromone.

Together, the findings that (a) more females tend to attack leg-ablated males, (b) pre-copulation attack rates did not differ between quiescent and non-quiescent females, and (c) quiescent females attacked less often post-copulation indicate that quiescence induced during copulation via cuticular organs located on the legs might benefit male *R. rabida* fitness by allowing him to escape injury and find additional mates.

When conducting behavioral experiments using animals that experience a manipulation by the experimenter such as an ablation, it is crucial to show that the ablation treatments did not affect the animals' behaviors outside of those being tested.

Any differences in behavior due to the ablation itself could alter the results in a way unrelated to the study objectives. As stated above, the manipulated males were able to court normally and did not differ among treatments in the latency to court, duration of courtship, or latency to copulate. However, I did observe a difference in the duration of copulation for males between the ablated pedipalps and ablated fangs treatments (**Figure 7**), but I believe that this is due to the males becoming disoriented and "frustrated" while attempting to copulate. In a previous study, male *R. rabida* behaved abnormally when they were unable to insert pedipalps into the female (Rovner 1971). In that study, Rovner submitted males to a variety of treatments where he modified them by removing one or both of their pedipalps. He also tested the male behaviors when the male was unmanipulated and paired with a female that had her epigynum (the female reproductive organ) sealed. When modified males could not complete the insertion they regularly became disoriented, continued to court, made rapid position changes (both side-to-side and forward-and-back), and deposited silk across the female's legs ("tying down" behavior). Disorientation, when the male rotated his position on top of the female to face either with the female (180 degrees from normal mating position) or at a right angle to the female, occurred most often with males lacking both palps. When a male with functional pedipalps was paired with a female that had her epigynum sealed the male often performed a "pseudo-insertion." Pseudo-insertions are the expansion of the male haematodochal sac without being inserted in the female epigynum; occasionally this is accompanied by the male raising the

expanded pedipalp dorsally and lowering it with the haematodochal sac collapse (Rovner 1971).

In the present study, most of these unusual male behaviors were observed. All the treatment manipulations and controls had disoriented males and males that courted mid-copulation – though courtship was seen only after dismounting and staying within reach of the female. Rovner (1971) did not observe these abnormal behaviors with unmanipulated spiders. Even though these behaviors were seen in all the treatments, they were performed more often in the ablated pedipalps treatment (personal observation). The males with ablated pedipalps, having been incapable of insertions, may have ceased copulation early compared to the other treatments. They may have ceased copulation due to fatigue of the muscles involved in haematodochal sac expansion after multiple attempts to insert (Rovner & Wright 1975) or they may have been unable to feel the female's epigynum and, in combination with low/no proprioception from their pedipalps (Rovner 1972, Sentenská et al. 2017b), dismounted the female to conserve energy. The tying down behavior was not observed in any of the current study trials. Another male behavior may have also contributed to the significant difference in copulation durations between the ablated pedipalps and ablated fangs treatments – pedipalpal moistening. Pedipalp moistening, when the male raises its pedipalp to his chelicerae after an insertion, functions to lubricate and moisten the haematodochal sac for proper unfolding and folding of the sac and often occurs between insertions in *R. rabida* (Gering 1953, Rovner 1972, Eberhard & Huber 2010). In the fang-ablation group, the fangs were glued into the cheliceral furrow to ablate the

fangs. On occasion the chelicerae would become glued together if the spider held his chelicerae together before the glue had set. This could have led the male to be unable to properly moisten his pedipalps and taken a longer time on average, even by just a few seconds, between insertions. If this were the case, then after dozens of insertions per pedipalp (Rovner 1972, this study), the males could have easily added enough time to make the difference between the males of ablated pedipalps and ablated fangs treatments significant.

Lastly, the only significant difference in copulation duration is between these same two ablation types (fangs and pedipalps), and neither were significantly different from the control or other treatment types in terms of copulation duration. Further, this study was focused on the female quiescent state post-copulation and there is not a significant difference between these two treatments in those results (**Figure 8**), in the number of females attacking their partners (**Figure 10**), or in the number of attacks among treatments.

Objective 3

Results from the homogenate trials showed the pheromone was not volatile and that palpation by the male likely plays a role in inducing females into quiescence. The finding that none of the females exposed to homogenate via filter paper became quiescent revealed that for the pheromone to be effective, it must be applied directly to the female. However, since direct application of some control solutions also induced quiescence, it appears that tactile pressure alone may also play an important role in inducing quiescence.

Previous observations by Rovner (1971, 1972) suggested that tactile pressure may play a role, but the results of the experiments described here make a stronger case for it. Rovner (1971) was able to induce females that had just mated to rotate their abdomens as if still in copula before they resumed their normal activity. He was even able to stimulate females to re-assume mating position and rotate their abdomens as if in copula after they had become active post-copulation. In both instances, he used a thin paint brush handle and applied a small amount of pressure on the posterior cephalothorax and anterior abdomen of the female. In another paper, Rovner (1972) suggests (without data) that the female's quiescent, or "inactive", state was induced by the male pinching, but not biting, the female with his chelicerae prior to dismounting. He states the cheliceral pinching was "obviously a method of insuring escape from the female since the female was inactivated for several minutes by this single harmless action." The female would become so "inactivated" she could be dragged a short distance before the male would finally let her go and fully separate himself from her. However, no data are provided for this behavior in the paper (Rovner 1972) and this was merely a hypothetical explanation.

In the mating trials, I observed the same cheliceral pinches that were regularly accompanied by the male also performing a "flailing" behavior – lifting and extending of all legs as if walking or running in place – that varied in speed and duration (this was not quantified). The male dragging the female a short distance also occurred on occasion with the female remaining in the dragged position after the male walked away. Importantly, the cheliceral pinches did not occur with every female that became

quiescent. Further, at least one male in the ablated fangs treatment had chelicerae unintentionally glued together and was unable to pinch the female during copulation but still induced her into quiescence.

In the homogenate trials, I found only three (out of six) of the treatments resulted in females becoming quiescent. These treatments had either the proteinaceous buffer or male homogenate in buffer applied directly to the female with a brush. This behavior could have been the result of the pressure from the brush touching the female as in Rovner (1971) or similar to the pressure from the male cheliceral pinches as in Rovner (1972). However, if pressure alone induced the quiescent state, I would have expected females to become quiescent after having the DDI water directly applied to them with the brush as well. Of the homogenate treatments that did put females in the quiescent state, a maximum of 57% of them in one treatment entered the subdued state (Brush with Buffer Control, **Figure 12**). In contrast, up to 100% of the females in the mating trials became quiescent (ablated fangs, **Figure 8**) among the different treatments. This difference in the efficacy of inducing the quiescent state between the paintbrush alone and the live male in the mating trials strongly suggests that the tactile pressure alone is not as effective as the pressure plus the chemical transferred by the male. Additionally, in the mating trials, only 27% of females became quiescent when paired with males that had ablated legs. The males' pedipalps and chelicerae were completely unmanipulated in this treatment so if pressure or cheliceral pinching were the cause of quiescence, I would likely have seen a higher percentage of females becoming quiescent when paired with leg-ablated males. With these findings from the

male homogenate trials combined with those previously discussed from the mating trials, I propose that there may be several, potentially interacting, mechanisms to result in the female quiescent state.

Assuming there are multiple factors involved in inducing females into the quiescent state, I would have expected more subdued females exposed to homogenized male legs and bodies than to the buffer alone, but that was not the case (**Figure 12**). There is the possibility the BSA proteins in the buffer were helping the female recognize a "mate" and the pressure from the brush did its part to induce quiescence. On the other hand, the homogenates being no more effective than the buffer alone could have been due to a number of possible scenarios. Homogenizing the entirety of the male's legs and body could have diluted or inactivated the putative chemicals rendering them less effective. Chemicals from the male's tissues also could have been released that caused the female to behave differently. That is, they did not inactivate the quiescence-inducing chemical, but the female may have simply had a different reaction to a new stimulus. Another possibility is the chemical is produced or mixed just before it is needed like that of the bombardier beetle (Arndt et al. 2015) and the homogenization did not allow for the males to adequately synthesize the compound.

The different rates of quiescence between the mating trials and the homogenate trials is interesting because of the different aspects of female manipulation that are highlighted in each experiment. The mating trials highlight the use of a semiochemical, and the homogenate trials highlight the possibility of male palpation being a factor in inducing the female state. Another interesting result is the difference in quiescent

durations between the experiments. The fact that the females in the homogenate trials were quiescent for so much longer (224-519 sec.), on average, than the females from the mating trials (51-171sec.) was an unexpected result. One possible explanation for this result is that the absence of a male in the arena creating vibrations through the substrate allowed them to remain in the quiescent state – the female was not stirred back into an active state. I find this unlikely though, since during the mating trials I kept the males from moving around the arenas by placing a small plastic cup over them post-copulation. Once cupped, the males would occasionally begin courting again in the cup, but many remained relatively still, so vibrations in the mating trials also would have been minimal.

On multiple occasions in the homogenate trials, I observed females that seemed only partially stunned. After having had solutions applied to them, the females would continue walking in the arena, but without using their fourth, and sometimes third, pair(s) of legs. These females would not become quiescent but seemed to lose the use of their back legs while walking. Spiders have many chemosensory receptor hairs on their legs (Tichy 2001, Ganske & Uhl 2018) so it is possible the brush accidentally touched the females' legs in these trials, leading to this more localized response. Homogenate or control solutions were applied to the posterior cephalothorax and anterior abdomen following the methodology of Rovner (1971, 1972) who noted that males regularly brushed their legs on and palpated that area of the female during copulation. After having observed this strange effect of only some legs being stunned,

future experiments should include the application of these solutions to the female legs as well.

The results of the homogenate trials are somewhat difficult to interpret. I was unable to test additional females and the solutions used were rather crude (with the complete homogenization of bodies and legs). The experiment provided some unexpected results and will be useful in designing a follow-up experiment in the future. Studies that aim to understand the mechanism leading to induced female quiescence by applying a solution to a female *R. rabida* should consider other possibilities than male homogenates. Some considerations could include using a cuticle wash that only extracts the chemicals from the exterior of the legs and the cuticular organs, a cuticle wash of males exposed to female silk and pheromone cues, or contacting the female directly with the dissected legs and pedipalps of a male instead of a solution. Another possible consideration for either an experiment with mating trials or direct applications would be to ablate the chemoreceptors on females. This could be done easily by anesthetizing the female and clipping the receptor hairs on her legs before the trials. If needed, these ablated females could be used in a repeated measures experimental design since the receptors do not grow back between molts and, assuming mature females are used, the females would not be molting again. Such an experiment may be most useful in determining female susceptibility to quiescence or further identification of a male pheromone, if a good pheromone candidate is found.

Objective 4

Results from the GC-MS analyses were largely inconclusive. Of the compounds that returned high library matches from GC-MS (**Table 2**), none appear to be good candidates as a pheromone to induce quiescence in female *R. rabida*. I propose a few options to consider if a study like this were to be conducted again. It is possible that the males do not produce the chemical until they encounter a female or her pheromones as proposed above in the discussion of the homogenate trials (bombardier beetle: Arndt et al. 2015). The spiders used in this analysis were all kept in individual containers and were not exposed to conspecifics at all from the time of their capture as juveniles to their sacrifice for the analysis. If males do only begin to synthesize the compound after encountering a female, then the males used here would have not had any time to produce it since he was kept separated from all females. If this is the case, then exposing a male to the pheromone-laden silk of a female just before anaesthetization and dissection for GC-MS analysis may be enough to solve this issue. If the presence of a female is required, flash freezing the pair mid-copulation may be needed (Poy et al. 2020).

Extracting the putative compounds may not require the full processing done here. For this study I dissected the legs, venom glands, and pedipalps (where applicable) from the body and inserted all the samples into a hexane and acetone solution before grinding the samples to release candidate compounds. Future analyses may only require sacrificing the spider and soaking its entire body in solution to extract the cuticular compounds (Adams et al. 2021). The additional processing could release unnecessary compounds from other organs in the spider's body that could mask or dilute the target

compounds. My data from the mating and homogenate trials also suggest no need to dissect the venom glands or pedipalps for separate analysis of those organs. If a good candidate compound is found, then electrophysiological studies could be conducted in addition to behavioral studies to confirm the effect of the compound (Tichy et al. 2001, Xiao et al. 2010).

Broad Implications

Studying behavior with experiments like what I have reported here are best understood when considering the four questions presented first by Tinbergen (1963). Tinbergen's four questions are regularly categorized into levels of questions: proximate (how) and ultimate (why). The proximate questions are concerned with how a behavior works physiologically (causation; how is the behavior constructed?) and how it changes over an individual's lifespan (ontogeny; how does the behavior develop?). The ultimate questions analyze why a behavior evolves (function; what is the utility of the behavior?) and the long-term development of the behavior (evolution; what was the original purpose of the behavior?). Here, I touch on three of Tinbergen's questions, both proximate and one ultimate. The SEM work looked into the development of the cuticular structures (do only mature males have the cuticular structures?). The mating trials were used to observe the adaptive value of males inducing females into quiescence (why do males induce females into the quiescent state?). Lastly, the homogenate trials and attempt to identify the pheromone involved by using GC-MS analyzed the mechanism (how is the quiescent behavior caused?).

Observing minute animal anatomy with SEM can reveal important ontogenetic developments of organs like the pits found on the legs of *R. rabida* with simple comparisons and relatively few samples. If the spiders have these pits their entire lives, it is possible that the spider would need them throughout their lives as well. The function of the pits would then be useful in other contexts apart from mating and that function would need to be identified. However, if the organs develop from one life stage (instar) to the next, a more specific purpose for them could be presumed even if the development required growth over two or three instars. Further SEM studies are needed to quantify cuticular pits of *R. rabida* and additional TEM studies will help determine product compounds of any glands associated with them. Identifying the location of the structures and organs emitting the chemical will lend information to the function of the structure when compared to the animal's behavior. Studying these aspects not only help us understand the behavior and ecology of the focal animal system but lend information to other animals' behavior, ecology, and development.

Behavioral studies like the mating trials are important for understanding sexual cooperation, sexual conflict, communication, and the discrete factors of a mating system. Specifically, in the mating trials, I was able to evaluate the adaptive value, or function, of the female quiescent state induced by males. Quiescent females were less likely to attack males post-copulation, the adaptive value of which is presumably to help the male escape injury or cannibalism. In these trials I used modified males and unmodified females that were still able to behave normally, outside of the induction of quiescence, and females were still able to reject a male if she did not find him attractive.

With that, we see that males are not forcing copulations and only trying to avoid injury and being cannibalized after mating which begs the question if this behavior evolved from sexual cooperation or sexual conflict. At this stage I think it is too early to tell because we do not know if the female gains or loses any benefits from being stunned.

Experiments on systems utilizing a chemically mediated behavior should use a combination of chemical analysis, such as GC-MS, behavioral tests with live animals, and electrophysiology techniques whenever possible. While doing these tests individually can be useful, the full mechanism cannot be identified without knowing the chemicals, receptors, sensitivities of the receptors, and overall behavioral outcome involved. The GC-MS and homogenate trials conducted here, even being preliminary, show that there does not have to be just one mechanism used to induce quiescence which, to my knowledge, has not been considered yet. The modified methods of chemical extraction and application to females discussed previously and the addition of electrophysiological experiments with larger sample sizes will help solidify the idea of a two-factor mechanism.

On induced quiescence specifically, future research should consider the aggressiveness of the focal species in comparison to closely related species in the same or similar habitats. As it stands, identifying whether sexual cooperation or sexual conflict drives the evolution of male manipulative behavior is very difficult and female aggression comparisons may be a way to do that. For instance, the dichotomy of aggressive behaviors of the females may have put different selective pressures on the males of the species leading to different strategies of aggressive female (injury and

cannibalism) avoidance while other pressures remain relatively the same. With such a scenario I would hypothesize the more aggressive species to see more sexual conflict in the mating system and perhaps a more "extreme" avoidance strategy from the male. The female then, in turn, may evolve to reject the male avoidance strategy and lead to a coevolutionary arms race between the sexes. I would then hypothesize the lesser aggressive species to display more sexual cooperation in this hypothetical scenario. The females, while still being fairly aggressive, could be selecting for high quality males but inadvertently also selecting for males that can effectively induce quiescence. This would require the male ability to be heritable.

If the male ability to induce quiescence is heritable, then females could benefit from being subdued by her mate. That is, her sons would be more likely to pass her genetics to future generations because they would be more likely to induce female quiescence in their mates. This, however, presupposes that males also vary in their ability to induce the quiescent state and that females are all equally susceptible to entering the state. We, of course, do not know if this is true at this time but some very interesting studies could be conducted to find out.

Variation in *R. rabida* female susceptibility to male chemical manipulation, and the relative cost or benefit of this behavior is unknown at this point. Being unresponsive could leave them open to being easy prey or to having a lesser quality male mate with them while they are still quiescent (Persons 2017). On the other hand, remaining very still following copulation could serve to enhance fertilization or other physiological processes. Future studies should measure offspring number and viability of females that

are stunned during copula and those that are not. This will help to illuminate whether the manipulation from the male provides an evolutionary advantage to the female as well as the male.

In this study I found that male *Rabidosa rabida* wolf spiders are inducing a state of quiescence in their female mates, the induction of quiescence is possibly achieved through two or more interacting mechanisms performed by the male, and females are less likely to attack a male post-copulation if she was quiescent while and after he dismounted. A male's ability to induce females into a less aggressive state can be advantageous for him if he is able to sire more offspring but we cannot conclude whether this is sexual conflict or cooperation. The complete mechanism(s) behind male induced female quiescence are unknown and, in some cases, may be more complicated in one species versus another. The males of *A. aperta* are able to induce the state in females from a distance (Becker et al. 2005) so one mechanism, volatile pheromones, may be involved while the current study suggests that at least pheromone and palpation are needed. The fact that males can manipulate females in such a way raises questions of whether these behaviors and physiological reactions are beneficial to both sexes (sexual cooperation) or only to the male (sexual conflict) and the determination of cooperation or conflict may be system dependent. However, by approaching behavioral studies of male manipulation with the proximate and ultimate questions in mind, we can better understand the influence of sexual cooperation and sexual conflict. Similar quiescence behavior exists throughout the spider phylogeny, and although it varies widely in how and why they evolved, findings from this study can help understand

patterns seen across species. Further study is needed to confirm these findings with larger sample sizes, to untangle the relative roles of tactile pressure versus pheromone transfer, and to investigate possible costs and benefits of aggression/cannibalism to both the male and female to determine whether this behavior should be considered sexual conflict or cooperation. Undoubtedly, disentangling the evolution of male manipulation of female mates will require much more work, and spider systems offer unique opportunities for such studies.

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Figure 1

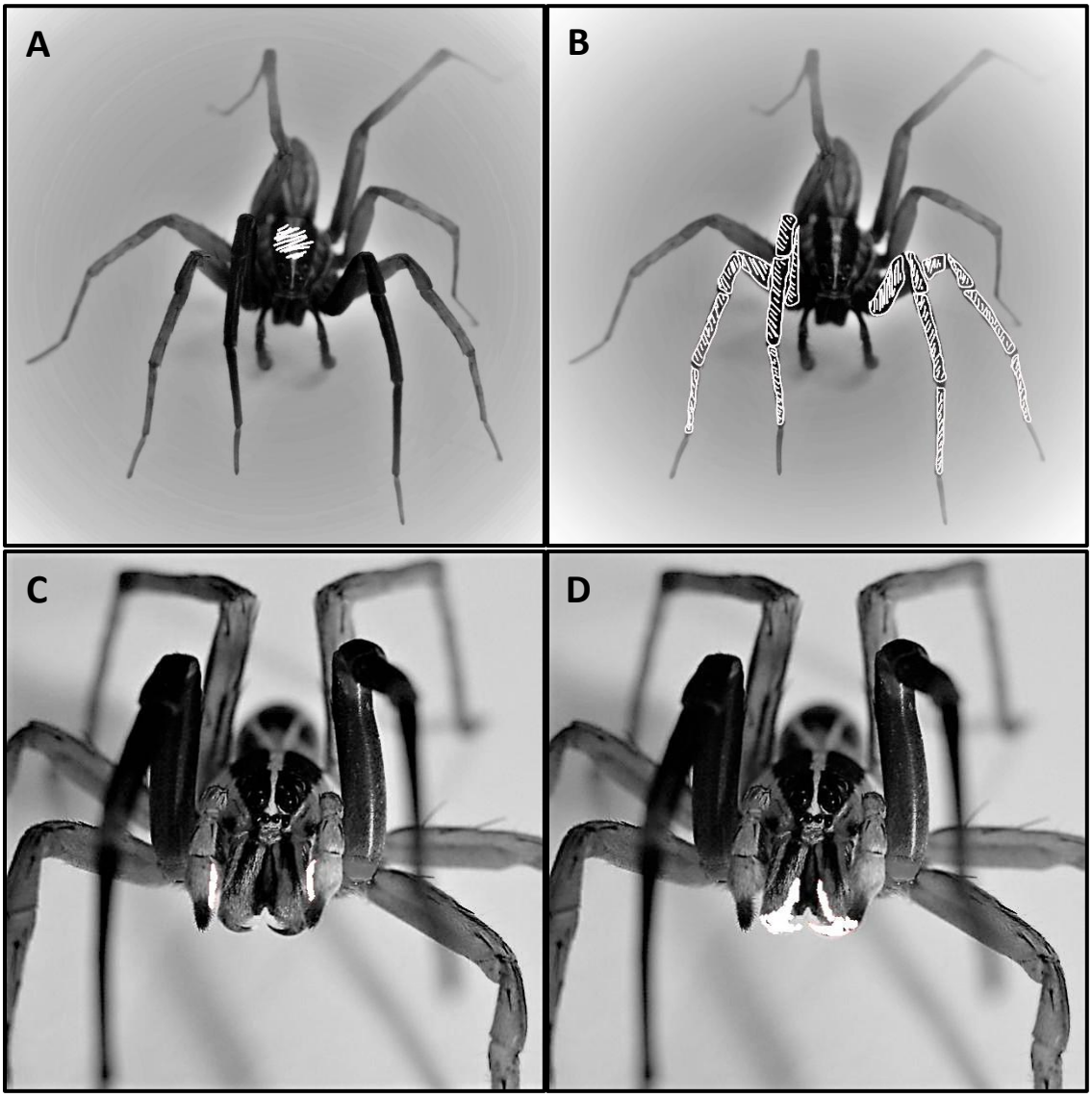

Figure 1a-d. Spider ablations for the mating trials. The white coloration indicates the region of the male body that glue was applied to for the ablation treatment. A) Ablation control, B) ablated legs, C) ablated pedipalps, D) ablated fangs. Figures A and B (Schoenberg 2019). Figures C and D (Brett Tyler 2008) modified by D. Schoenberg.

Figure 2

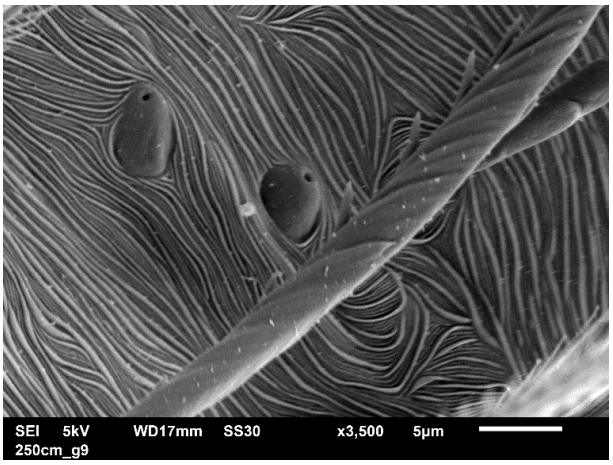

Figure 2a. Scanning electron micrograph of juvenile *R. rabida* male metatarsus

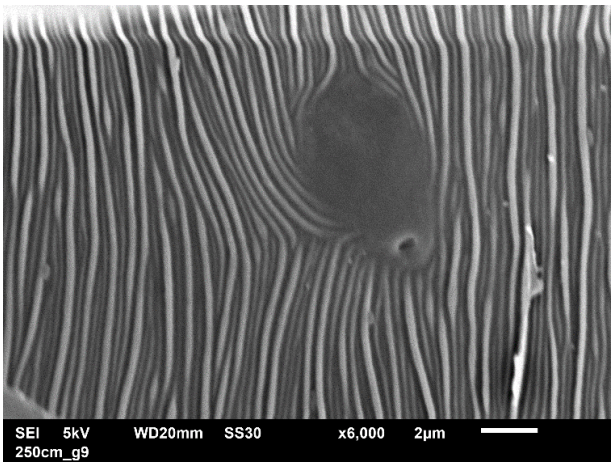

Figure 2b. Scanning electron micrograph of mature *R. rabida* female tibia

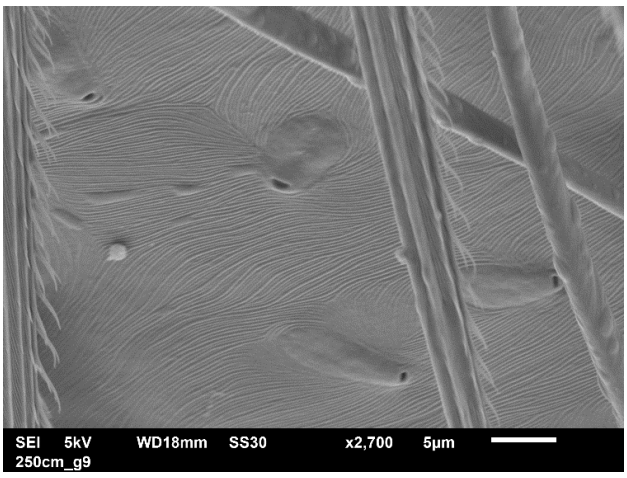

Figure 2c. Scanning electron micrograph of mature *R. rabida* male femur

Figure 3

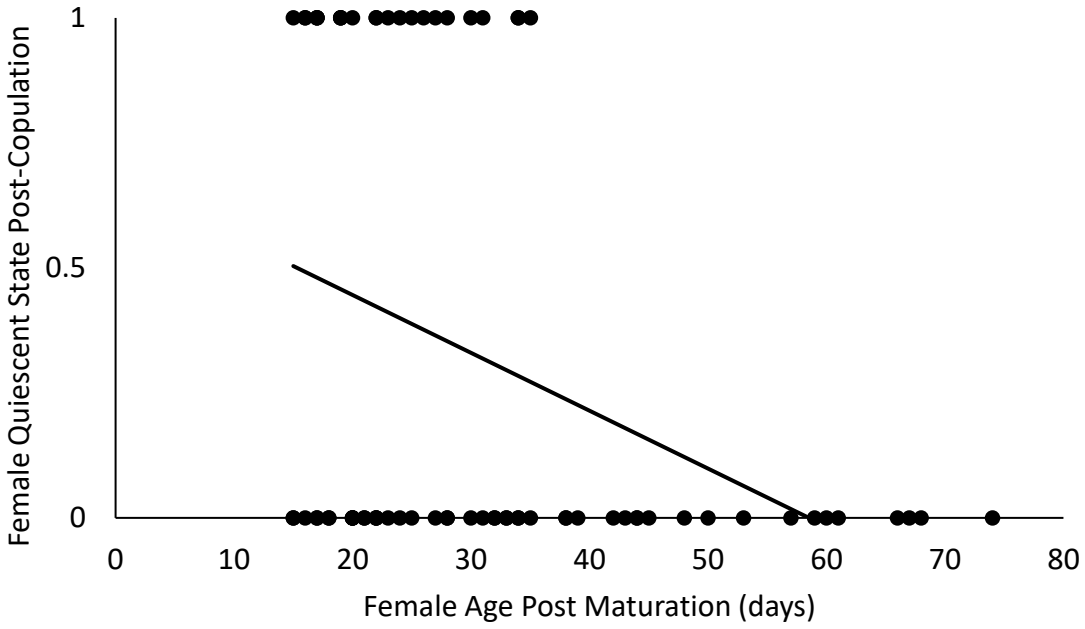

Figure 3. Female quiescent state (yes/no) post-copulation with only the 2019 data (n = 73). On the Y-axis, “0” indicates that the female was not quiescent and “1” indicates that the female was quiescent post-copulation. The females age ranged from 15 to 74 days old post maturation. The trendline shows that as females aged, they were less likely to enter quiescence.

Figure 4

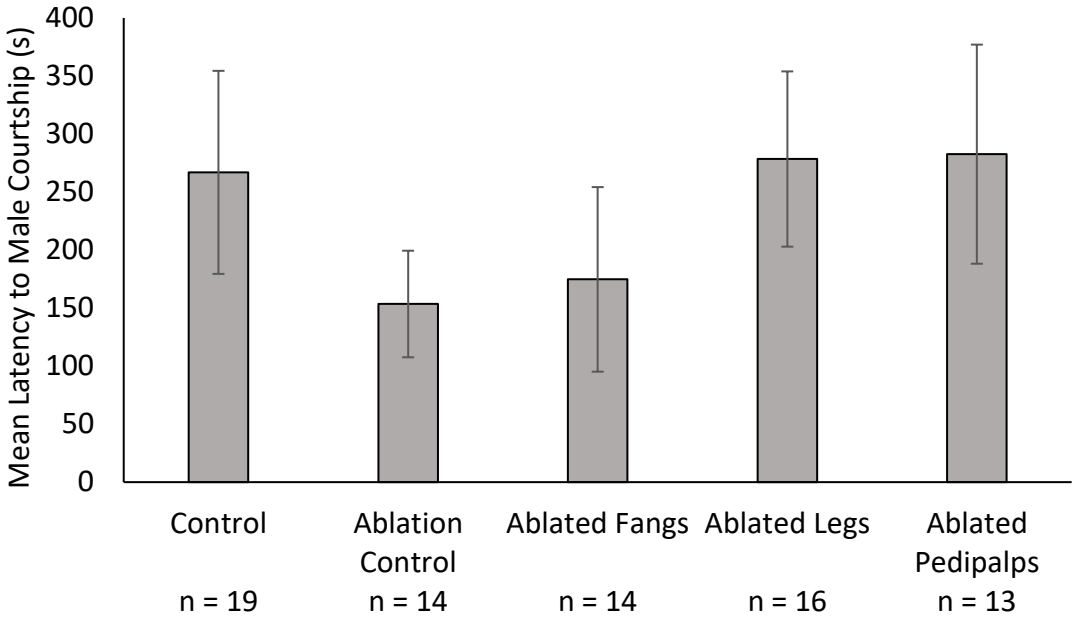

Figure 4. Mean male latencies to begin courtship (s) +/- standard error. Data from 2019 and 2020 trials (n = 76).

Figure 5

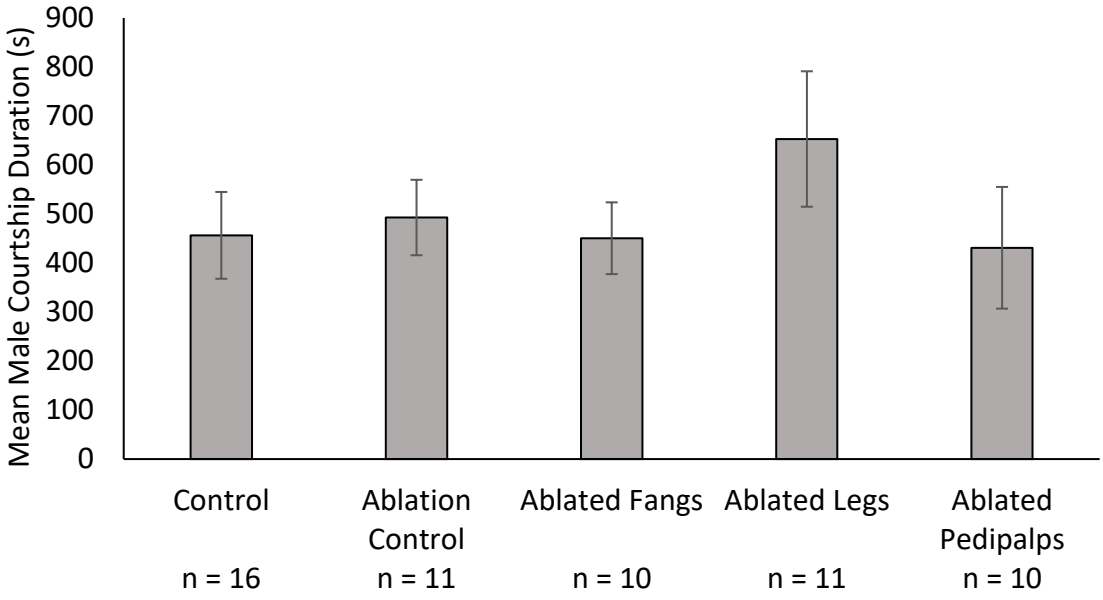

Figure 5. Mean durations of male courtship (s) +/- standard error. Data from 2019 and 2020 trials (n = 58).

Figure 6

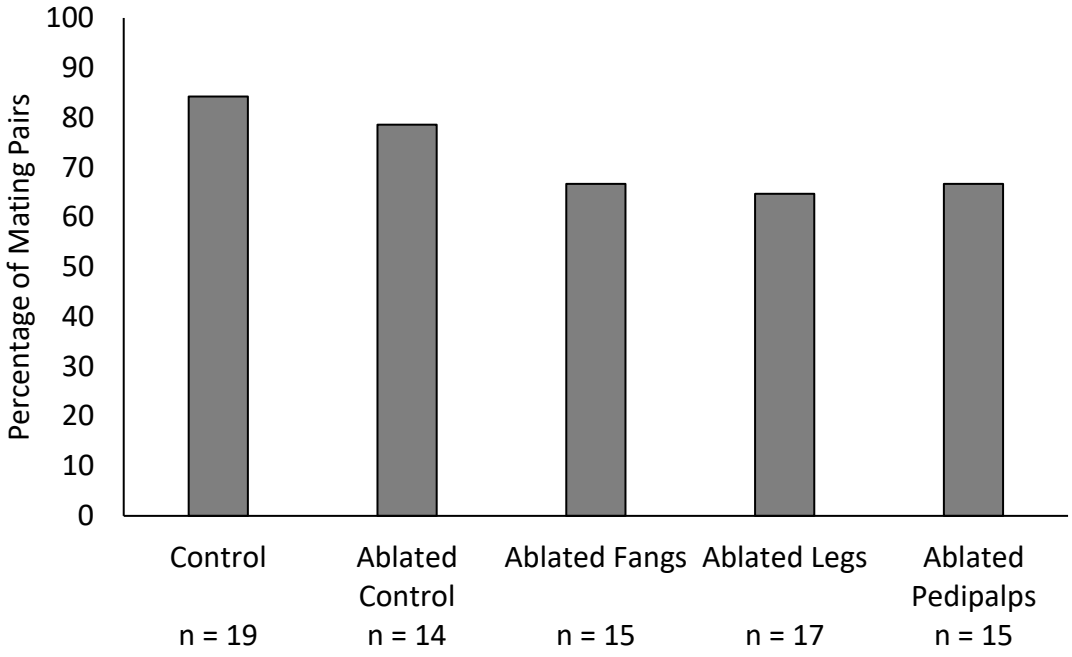

Figure 6. Percentages of pairs that copulated with the 2019 and 2020 data combined (n = 80). Of the combined 2019 and 2020 trials, eighty-four percent of the Control (n = 16/19), seventy-nine percent of the Ablated Control (n = 11/14), sixty-seven of the Ablated Fangs (n = 10/15), sixty-five percent of the Ablated Legs(n = 11/17), and sixty-seven percent of the Ablated Pedipalps (n = 10/15) pairs mated.

Figure 7

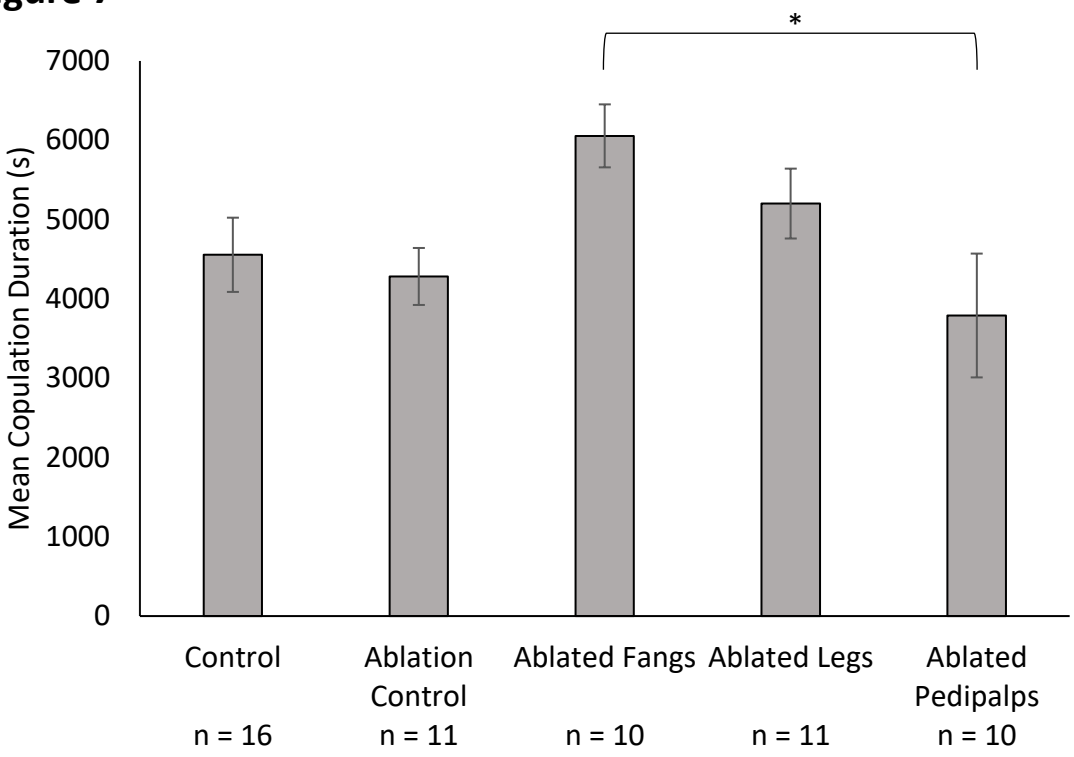

Figure 7. Mean copulation durations (s) +/- standard error. Data from 2019 and 2020 trials (n = 58). The asterisk (*) denotes a statistically significant difference between the Ablated Fangs and Ablated Pedipalps treatments.

Figure 8

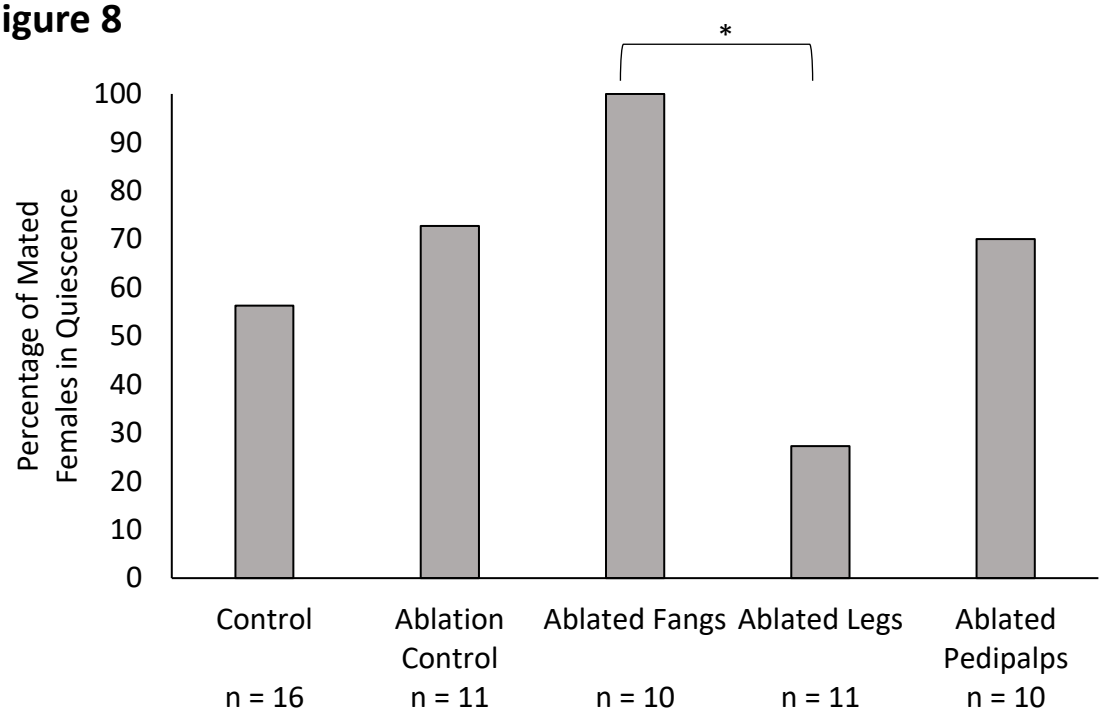

Figure 8. Percentage of females in the quiescent state post-copulation per treatment type. Data from 2019 and 2020 trials (n = 58). Of that pairs that mated fifty-six percent of Control (n = 9/16), seventy-three percent of Ablation Control (n = 8/11), one hundred percent of Ablated Fangs (n = 10/10), twenty-seven percent of Ablated Legs (n = 3/11), and seventy percent of Ablated Pedipalps (n = 7/10) females were quiescent post-copulation. The asterisk (*) denotes a significant different between the ablated fangs and ablated legs treatment groups.

Figure 9

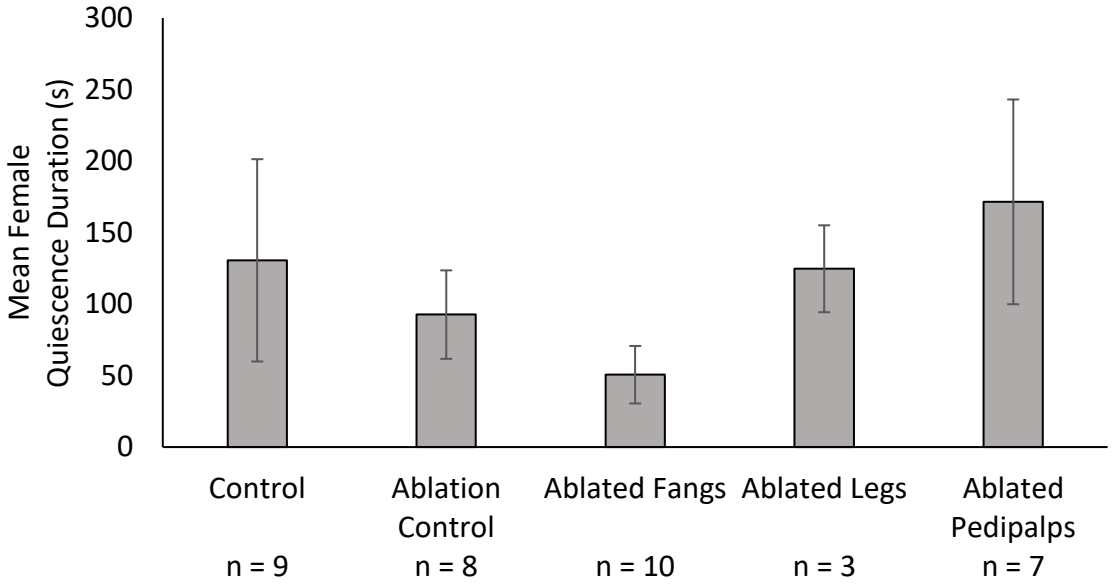

Figure 9. Mean female quiescence duration of females in the quiescent state post-copulation per treatment type (n = 37) +/- standard error. Data from the 2019 and 2020 trials.

Figure 10

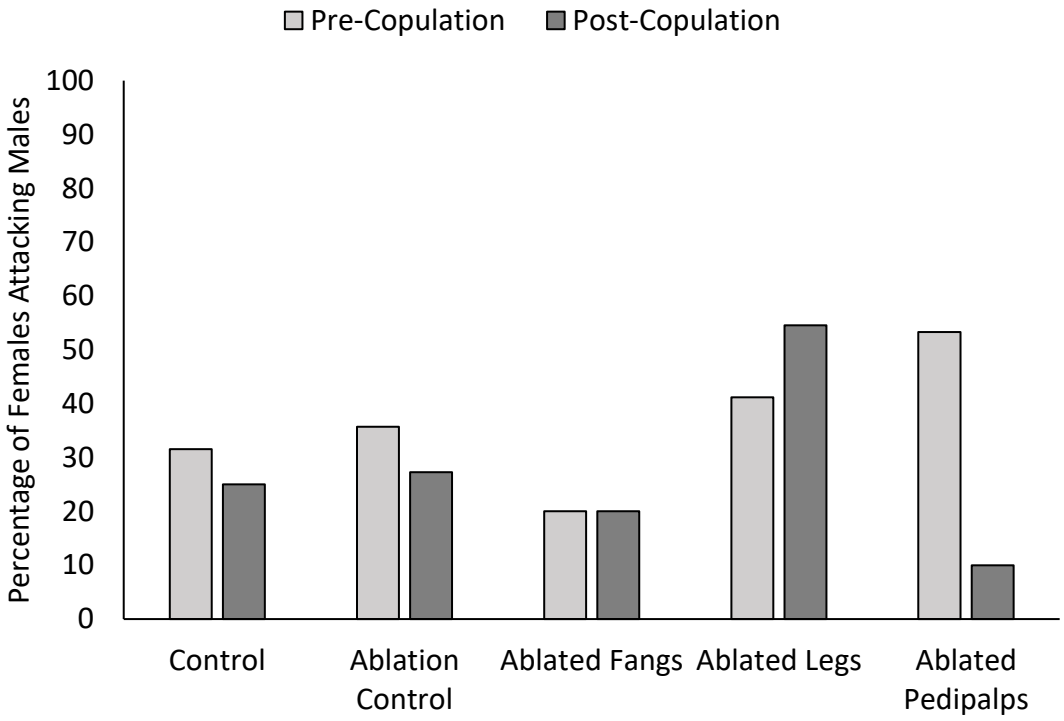

Figure 10. Percentage of females that attacked their male partner pre-copulation (n = 80), light grey, and post-copulation (n = 58), dark grey, per treatment. For the females that attacked their partners pre-copulation thirty-two percent of Control (n = 6/19), thirty-six percent of Ablation Control (n = 5/14), twenty percent of Ablated Fangs (n = 3/15), forty-one percent of Ablated Legs (n = 7/17), and fifty-three percent of Ablated Pedipalps (n = 8/15). Of the females that mated and attacked their partner post-copulation twenty-five percent of Control (n = 4/16), twenty-seven percent of Ablation Control (n = 3/11), twenty percent of Ablated Fangs (n = 2/10), fifty-five percent of Ablated Legs (n = 6/11), and ten percent of Ablated Pedipalps (n = 1/10). Data from 2019 and 2020 trials.

Figure 11

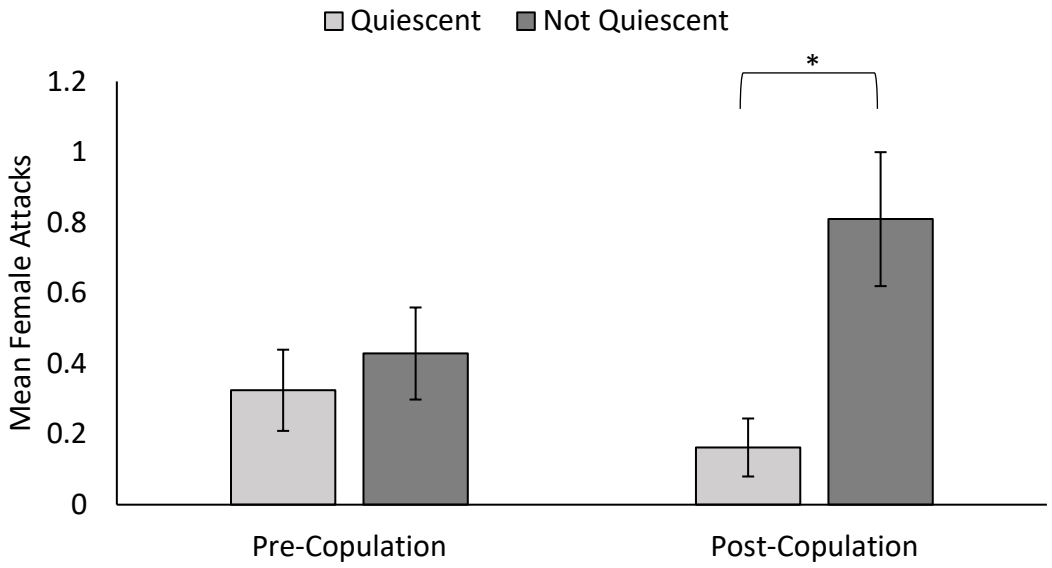

Figure 11. Mean number of attacks by females pre-copulation (n = 58) and post-copulation by their quiescent status (n = 58) +/- standard error. The asterisk (*) denotes a significant difference in mean number of attacks by females post-copulation.

Figure 12

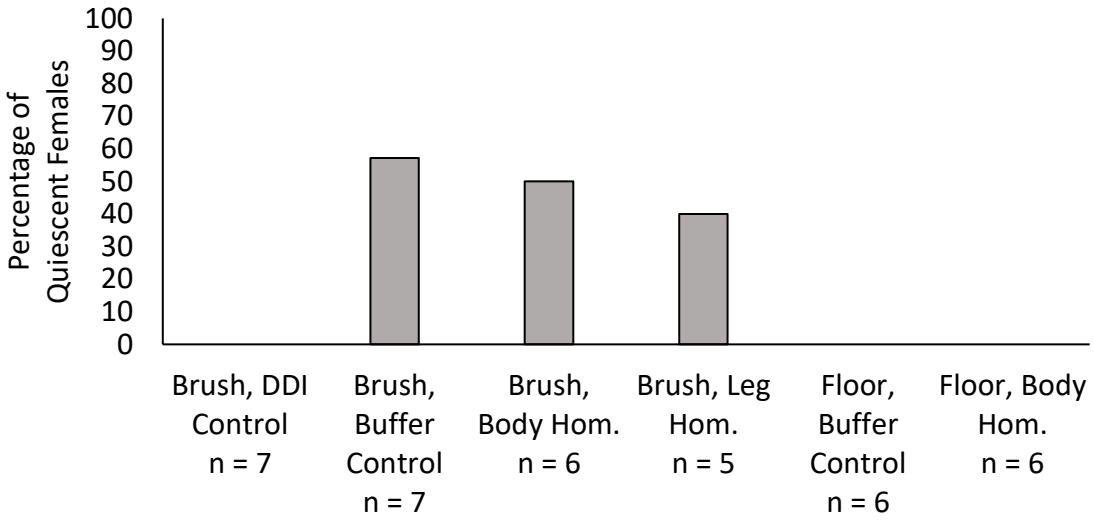

Figure 12. Percentages of females that became quiescent per treatment in the homogenate trials (n = 37). Fifty-seven percent of Brush with Buffer Control (n = 4/7), fifty percent of Brush with Body Homogenate (n = 3/6), forty percent of Brush with Leg Homogenate (n = 2/5) females became quiescent and stood in mating position when the respective solution was applied to their abdomen. No females became quiescent in the other three treatments.

Figure 13

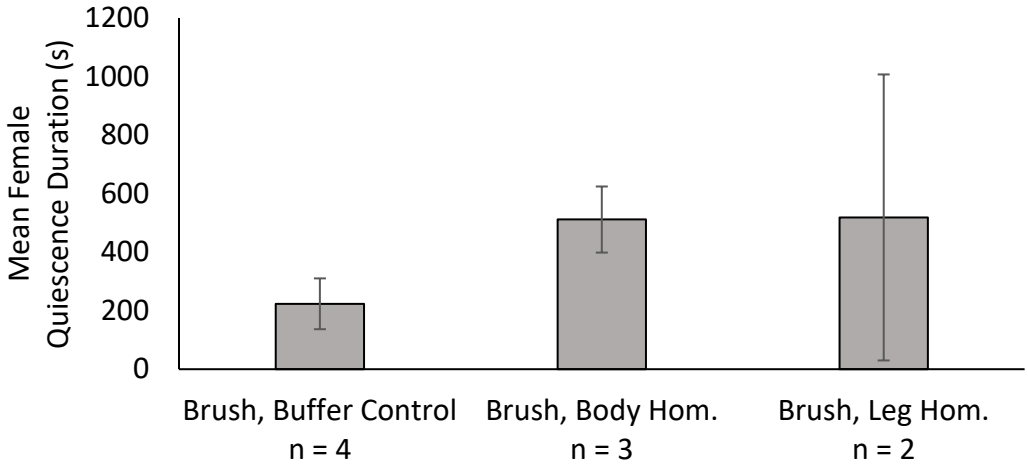

Figure 13. Mean durations of female quiescence by treatment (n = 9) +/- standard error.

Table 1. The total number of cannibalisms pre- and post-copulation per treatment. Data is from 2019 and 2020 mating trials.

Cannibalism	Pre-Copulation	Post-Copulation
Control	0	2
Ablation Control	1	0
Ablated Fangs	0	1
Ablated Legs	0	2
Ablated Pedipalps	1	0

Table 2. Summary of the GC-MS output from the bodies, legs, venom glands, and pedipalps of a juvenile female, juvenile male, mature female, and mature male *R. rabida.*

Sample ID	Compound	Class	Retention Time(min)	Library match
Juvenile Female Body	Dodecane	alkane	6.872	97
	1,3-bis(1,1-dimethylethyl)-benzene	alkylbenzene	7.505	95
	Tetradecane	alkane	8.869	96
	2,4-bis-(1,1-dimethylethyl)-phenol	substituted phenol	9.94	97
	Hexadecane	alkane	10.661	97

	n-hexadecanoic acid	fatty acid	13.42	99
	9,12 ocadecanoic acid	fatty acid	14.29	99
	Octadecanoic acid	fatty acid	14.37	89
	1-docosanol methyl ether	ether	15.29	98
	Butyl, 9,12-octadecadienoate	butyl ester	15.65	98
Juvenile Female Legs	N,N-dimethyl-2-aminoethanol	ethanolamine	3.39	78
	Methoxy, phenyl oxime	oxime	4.134	87
	1,3-bis(1,1-dimethylethyl)-benzene	alkylbenzene	7.505	96
	2,4-bis-(1,1-dimethylethyl)-phenol	substituted phenol	9.96	96
	9,12 ocadecadienoic acid	fatty acid	13.34	93
	n-hexadecanoic acid	fatty acid	13.41	99
	9-Eicosyene	alkyne	14.3	98
	9,12 ocadecanoic acid	fatty acid	14.29	99
	Cholesta-3,5-diene	cholesterylene	15.41	99
Juvenile Female Venom Gland	N,N-dimethyl-2-aminoethanol	ethanolamine	2.72	78
	1,1’-(1,2-ethanediyl)-bis-piperidine	piperidine	7.16	78
	1,3-bis(1,1-dimethylethyl)-benzene	alkylbenzene	7.52	93
	Cholesterol	Cholesterol	14.7	99
Juvenile Male Body	N,N-dimethyl-2-aminoethanol	ethanolamine	3.28	
	9,12 ocadecadienoic acid	fatty acid	14.28	99
	Pregn-5-ene-3,20-diol, (3, beta, 20S)	hormone	14.01	96

	Pregn-5,17(20)-dien-3-ol (3, beta, 17E)	hormone	13.92	70
	Cholesterol	Cholesterol	14.7	99
Juvenile Male Legs	N,N-dimethyl-2-aminoethanol	ethanolamine	3.11	
	Ornithine	non-proteinogenic amino acid	8.38	83
	9,12 ocadecanoic acid	fatty acid	13.35	95
	Pregn-5-ene-3,20-diol, (3, beta, 20S)	hormone	14.09	95
	Pentacosane	alkane	15.4	94
Juvenile Male Pedipalps	1,3-bis(1,1-dimethylethyl)-benzene	alkylbenzene	7.505	95
	2,4-bis-(1,1-dimethylethyl)-phenol	substituted phenol	9.97	94
	Cyclohexadecane	alkane	14.02	99
	2,2'-methylenebis(6-(1,1-dimethylethyl-4-methyl phenol	substituted alcohol	15.51	96
Juvenile Male Venom Gland	1,3-bis(1,1-dimethylethyl)-benzene	alkylbenzene		
	2,4-bis-(1,1-dimethylethyl)-phenol	substituted phenol		
	5-Octadecane (E)	alkane	14.03	98
	Butyl, 9,12-octadecadienoate	butyl ester	15.64	98
Mature Female Body	1,3-bis(1,1-dimethylethyl)-benzene	alkylbenzene		
	2,4-bis-(1,1-dimethylethyl)-phenol	substituted phenol		
	9,12 ocadecadienoic acid	fatty acid		

Mature Female Legs	1,3-bis(1,1-dimethylethyl)-benzene	alkylbenzene		
Mature Female Venom Glands	1,3-bis(1,1-dimethylethyl)-benzene	alkylbenzene		
	1-methyl-2, piperidinemethanol	piperidine	7.2	78
	1-docosene	alkene	15.69	95
Mature Male Body	Methoxy, phenyl oxime	oxime	3.96	83
	Pregn-5,17(20)-dien-3-ol (3, beta, 17E)	hormone	13.94	55
	Pregn-5-ene-3,20-diol, (3, beta, 20S)	hormone	14.09	95
	Sitosterol	steroid	15.01	99
	Pentacosane	alkane	15.48	97
Mature Male Legs	1,3-bis(1,1-dimethylethyl)-benzene	alkylbenzene		
Mature Male Pedipalp	Butyl, 9,12-octadecadienoate	butyl ester	15.64	98
	Squalene	poly unsaturated hydrocarbon	14.46	96
	13-docosenamide (Z)	fatty acid amide	14.1	93
	5-octadecene (E)	alkene	14	97
Mature Male Venom Gland	1-methyl-2, piperidinemethanol	piperidine	7.22	78